

A THESIS

entitled

ARACHIDONIC ACID METABOLISM BY BLOOD PLATELETS

by

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Submitted for the degree of Doctor of Philosophy

in

The University of Edinburgh

University of Edinburgh

1980



The work described in this thesis was performed by myself
(unless otherwise stated) in the Department of Pharmacology,
University of Edinburgh between 1976 and 1979.

Certain sections of the work described in this thesis have been published.

R. L. Jones, P. J. Kerry, N. L. Poyser, I. C. Walker and N. H. Wilson. The identification of trihydroxyeicosatrienoic acids as products from the incubation of arachidonic acid with washed blood platelets. *Prostaglandins*, 1978, 16, 583-589.

R. L. Jones, P. J. Kerry, N. L. Poyser, I. C. Walker and N. H. Wilson. Novel metabolites of arachidonic acid. In: *Chemistry, Biochemistry and Pharmacological Activity of Prostanoids* (S. Roberts and F. Scheinmann, eds.), Pergamon Press, 1979, p. 138-148.

I. C. Walker, R. L. Jones and N. H. Wilson. The identification of an epoxy-hydroxy acid as a product from the incubation of arachidonic acid with washed blood platelets. *Prostaglandins*, 1979, 18, 173-178.

I. C. Walker, R. L. Jones, P. J. Kerry and N. H. Wilson. An epoxy-hydroxy product from arachidonate. In: *Advances in Prostaglandin and Thromboxane Research*, Raven Press, in press.

ACKNOWLEDGEMENTS

I would like to thank Professor E. W. Horton for his help and kindness at all times. Particular thanks are also due to Dr. R. L. Jones and Dr. N. H. Wilson for their supervision and advice and Dr. P. J. Kerry (who was involved with me in aspects of the work described in Section 5 of this thesis) for his help and encouragement. Thanks are also due to Mr. C. G. Marr and Mr. K. Bell for their technical assistance.

Finally, I would like to thank Miss Angela Gray for her excellent typing of the manuscript.

The work described in this thesis was performed during the tenure of an MRC studentship to myself and an MRC Programme Grant to Professor E. W. Horton.

CONVENTIONS AND NOMENCLATURE

The basic 20-carbon skeleton of the prostaglandins has been named prostanoic acid. The correct chemical name of all prostaglandins, their metabolites and analogues can be derived by reference to this structural formula. These chemical names, although precise, are long and tedious to use; therefore for the major prostaglandins, trivial names are used.

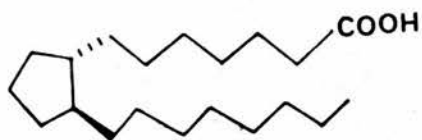
Nine series of natural prostaglandins have so far been described, designated by the letters A-I. The natural prostaglandins have an S-hydroxyl group (or hydroperoxy group in the case of PGG) at C-15 and contain a 13,14-trans double bond. The degree of unsaturation of the side chains is indicated by the subscript numeral (1, 2 or 3) after the letter. For all classes of prostaglandins, these bonds appear at the same position and possess the same stereochemistry. In addition to the 13, 14 double bond, members of the 2 series prostaglandins contain a 5,6-cis double bond. Members of the 3 series contain a third 17,18-cis double bond. The subscript α refers to the configuration of the C-9 hydroxyl group.

The reader is referred to the following table. From these examples, the trivial names of all common prostaglandins can be deduced. In drawings of chemical structures, stereochemistry is not implied unless specifically indicated; a thickened or dotted line

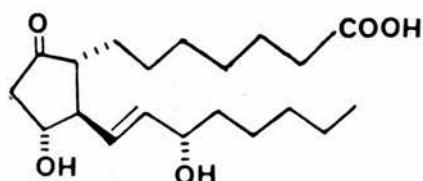
denotes a substituent located respectively above or below the plane of the paper.

Trivial Name

Systematic Name

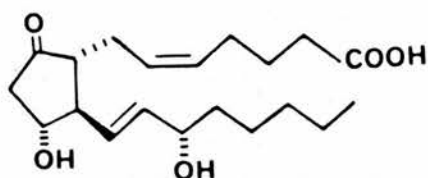


Prostanic acid



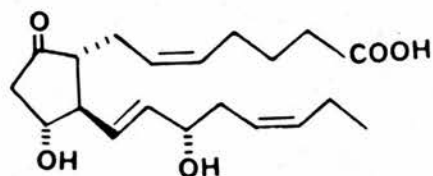
PGE₁

11 α ,15(S)-dihydroxy-9-keto-13trans-prostenoic acid



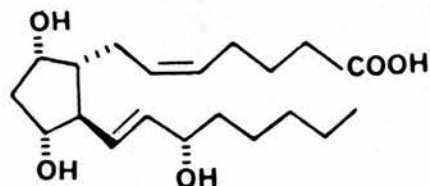
PGE₂

11 α ,15(S)-dihydroxy-9-keto-5cis,13trans-prostadienoic acid



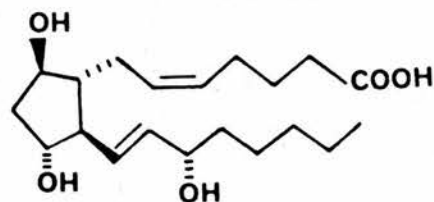
PGE₃

11 α ,15(S)-dihydroxy-9-keto-5cis,13trans-17cis-prostatricenoic acid



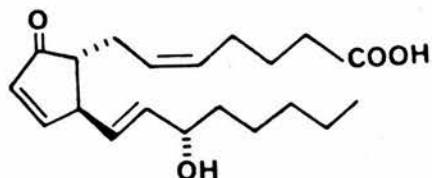
PGF₂ α

9 α ,11 α ,15(S)-tri-hydroxy-5cis,13trans-prostadienoic acid



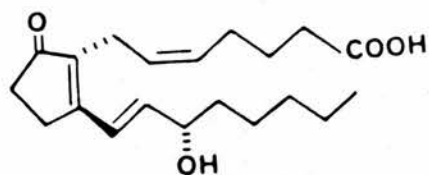
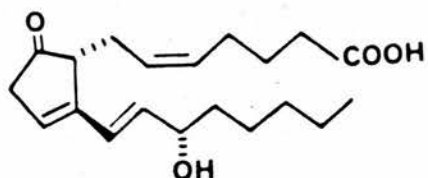
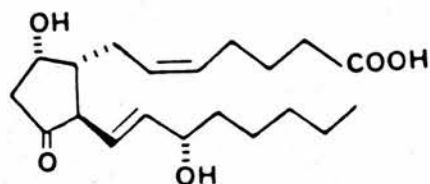
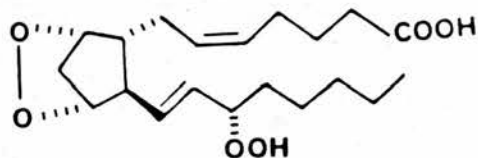
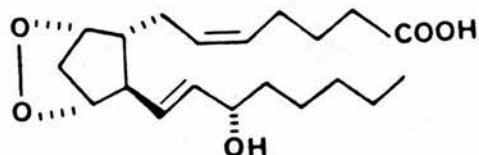
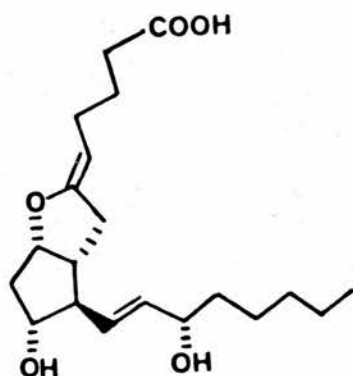
PGF₂ β

9 β ,11 α ,15(S)-tri-hydroxy-5cis,13trans-prostadienoic acid



PGA₂

15(S)-hydroxy-9-keto-5cis,10,13trans-prostatricenoic acid

Trivial NameSystematic NamePGB₂15(S)-hydroxy-9-keto-
5cis,8(12),13trans-
prostatricienoic acidPGC₂15(S)-hydroxy-9-keto-
5cis,11,13trans-
prostatricienoic acidPGD₂9α,15(S)-dihydroxy-11-
keto-5cis,13trans-
prostadienoic acidPGG₂15(S)-hydroperoxy-9α,11α-
peroxido-5cis,13trans-
prostadienoic acidPGH₂15(S)-hydroxy-9α,11α-
peroxido-5cis,13trans-
prostadienoic acidPGI₂11α,15(S)-dihydroxy-9-
deoxy-6,9α-epoxy-5Z,13-
trans-prostadienoic acid

ABSTRACT

The work described in this thesis is concerned with the metabolism of arachidonic acid metabolites by blood platelets.

Incubation of arachidonic acid with washed blood platelets (from human, horse, dog, cat and rabbit) revealed the presence of novel arachidonic acid metabolites in addition to those previously reported. Purification of the incubation extracts by liquid-gel partition chromatography followed by a gas chromatographic-mass spectrometric examination of a number of derivatives of selected chromatographic zones provided structural evidence for stereoisomers of two trihydroxy acids (8,11,12-trihydroxy-5,9,14-eicosatrienoic acid and 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid) and isomers of an epoxy-hydroxy acid (10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid).

As the formation of these metabolites was not inhibited by indomethacin but was inhibited by eicosa-5,8,11,14-tetraynoic acid, it was postulated that the metabolites were products of the platelet lipoxygenase pathway formed as a result of transformation involving 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid.

A range of pharmacological tests were applied to the newly identified arachidonic acid metabolites. Pronounced

activity was only observed with 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid in the chemotaxis assay.

CONTENTS

	<u>Page</u>
<u>SECTION 1 - INTRODUCTION</u>	1
1.1 Pathways of Arachidonic Acid and Dihomo- γ -linolenic Acid Metabolism	2
1.2 The Role of Arachidonic Acid Metabolites in Platelet Functions	17
1.3 Procedures for the Identification of Arachidonic Acid Metabolites	29
<u>SECTION 2 - MATERIALS AND METHODS</u>	39
2.1 Materials	40
2.2 Treatment of Blood	43
2.3 Incubation and Extraction Conditions	46
2.4 Preparation of Derivatives	49
2.5 Liquid Chromatographic Methods	52
2.6 Gas Chromatography-Mass Spectrometry	56

CONTENTS CONTINUED

	<u>Page</u>
2.7 Liquid Scintillation Counting	58
2.8 Ultraviolet Spectroscopy	59
2.9 Pharmacological Experimental Methods:	60
1. Platelet Aggregation	60
2. Prostaglandin I ₂ Production	61
3. Tissue Preparations	63
4. Chemotaxis	64
5. Blood Pressure Experiments	68
<u>SECTION 3 - THE INCUBATION OF ARACHIDONIC ACID</u>	69
<u>WITH WASHED BLOOD PLATELETS WITH THE DETECTION</u>	
<u>AND SUBSEQUENT STRUCTURE ELUCIDATION OF NOVEL</u>	
<u>METABOLITES - PART 1</u>	
3.1 Introduction	70
3.2 The Initial Examination of Platelet Incubates by Gas Chromatography-Mass Spectrometry	71
3.3 Separation of the Positional Isomers of the Trihydroxy acids by Liquid-Gel Chromatography	77

CONTENTS CONTINUED

	<u>Page</u>
3.3 and Examination of a Variety of Derivatives Cont of each by Gas Chromatography-Mass Spectro- metry	77
3.4 Factors Affecting the Formation of the Tri- hydroxy Acids	83
3.5 Concluding Remarks	86
<u>SECTION 4 - THE INCUBATION OF ARACHIDONIC ACID</u> <u>WITH WASHED BLOOD PLATELETS WITH THE DETECTION</u> <u>AND SUBSEQUENT STRUCTURE ELUCIDATION OF NOVEL</u> <u>METABOLITES - PART II</u>	89
4.1 Introduction	90
4.2 The Examination of the Two Least Polar Liquid- Gel Chromatographic Zones and the Detection and Examination by Gas Chromatography-Mass Spectrometry of a Molecule Analogous to the Proposed THETA Intermediate	91
4.3 Examination of Other Liquid-Gel Chromato- graphic Zones	105

CONTENTS CONTINUED

	<u>Page</u>
4.4 Concluding Remarks	109
<u>SECTION 5 - THE PHARMACOLOGICAL TESTING OF THE NEWLY IDENTIFIED ARACHIDONIC ACID METABOLITES</u>	112
5.1 Introduction	113
5.2 Results of Pharmacological Testing:	115
1. Platelet Aggregation	115
2. Prostaglandin I ₂ Production	117
3. Tissue Preparations	119
4. Chemotaxis	121
5. Blood Pressure Experiments	128
5.3 Concluding Remarks	130
<u>SECTION 6 - CONCLUDING REMARKS</u>	132
<u>REFERENCES</u>	138

SECTION 1

INTRODUCTION

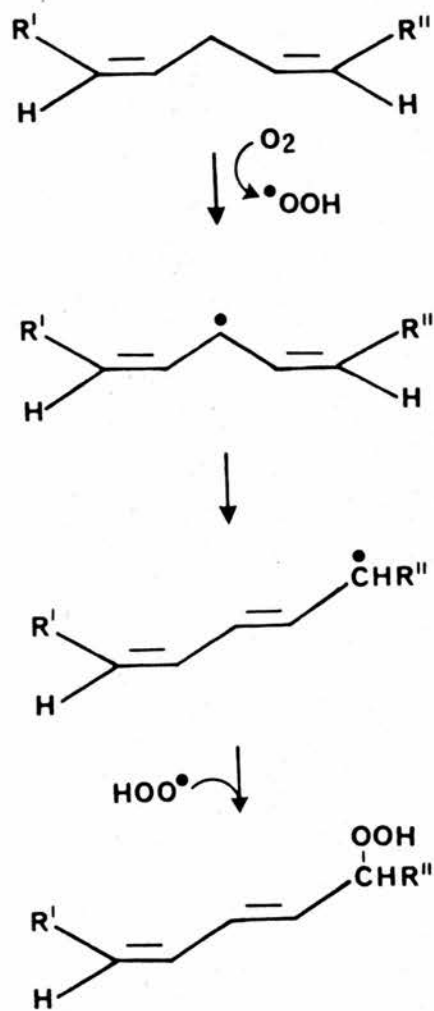


FIGURE 1.1 Proposed mechanism of lipid peroxidation

Introduction

The enzymic oxygenation reactions of all cis-5,8,11,14-eicosatetraenoic acid (arachidonic acid) and to a lesser extent all cis-8,11,14-eicosatrienoic acid (dihomo- γ -linolenic acid) represent an extensive area of biochemical and pharmacological research. The resultant products are implicated in a wide range of physiological processes.

For every lipoxygenase oxygenation type of reaction, the minimum requirement is the presence in the molecule of two cis double bonds separated by a methylene group. It has been proposed that the mechanism involves abstraction by oxygen of a methylene hydrogen atom to form a free radical that is stabilised by re-arrangement to a conjugated diene and that this stabilised radical combines with a hydroperoxy radical to yield the hydroperoxy acid (Figure 1.1) (Gurr and James, 1975). Assuming this mechanism, arachidonic acid can be expected to undergo peroxidation at six possible positions to yield products with a hydroperoxy group at C-5, C-8, C-9, C-11, C-12 and C-15. Dihomo- γ -linolenic acid can similarly be expected to undergo peroxidation at four possible positions with insertion of a hydroperoxy group at C-8, C-11, C-12 and C-15.

The rest of this subsection outlines the principal information that has been determined concerning enzymic oxygenation at each of these positions.

Oxygenation at the C-15 Position

Soyabean lipoxygenase is probably the most widely studied plant lipoxygenase enzyme. Four isoenzymes have been identified (Christopher and Axelrod, 1971; Christopher, Pistorius and Axelrod, 1972; Christopher, 1972 and Pistorius, 1974 - referenced within Bild, Ramadoss and Axelrod, 1977). They share a common requirement for the cis,cis-1,4-pentadiene system but differ in a number of ways including pH optima, the nature of secondary reactions they catalyse, the chemical nature of the substrate and positional specificity for the site of oxygen insertion.

Of these isoenzymes, soyabean lipoxygenase-1 is perhaps the most widely studied. Studies with this enzyme using linoleic acid as substrate (Hamberg and Samuelsson, 1967; Christopher, Pistorius, Regnier and Axelrod, 1972) have confirmed a positional specificity for attack at the $\omega 6$ position with a weaker specificity for the $\omega 10$ position. Hamberg and Samuelsson reported that substrates which contained three of four double bonds such as arachidonic acid or dihomo- γ -linolenic acid only formed products oxygenated in the $\omega 6$ position. Treatment

of arachidonic acid and dihomo- γ -linolenic acid with soyabean lipoxygenase-1 resulted in high yields (80-90%) of 15L-hydroperoxy-5,8,11,13-eicosatetraenoic acid and 15L-hydroperoxy-8,11,13-eicosatrienoic acid respectively.

Small amounts of 15-hydroxy-5,8,11,13-eicosatetraenoic acid and 15-hydroxy-8,11,13-eicosatrienoic acid have been identified following incubation of arachidonic acid and dihomo- γ -linolenic acid respectively with preparations of sheep vesicular gland (Hamberg and Samuelsson, 1967a; Wlodawer and Samuelsson, 1973). Trace amounts of 15-hydroxy-5,8,11,13-eicosatetraenoic acid were also identified following incubation of arachidonic acid with guinea pig lung homogenates (Hamberg and Samuelsson, 1974). These hydroxy compounds were presumed to be formed from the corresponding hydroperoxy compounds.

Recently, Borgeat and Samuelsson (1979) have reported the formation of 15L-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) following incubation of arachidonic acid with human polymorphonuclear leukocytes (PMNL). Interestingly, this product was detected only in human PMNL and not rabbit PMNL incubations. Although 15-HETE is synthesised by human PMNL, it was reported not to have any significant chemotactic activity for these cells (Turner and Lynn, 1978).

Oxygenation at the C-11 Position

The prostaglandins are a family of cyclopentane fatty acids. They have been found to occur naturally in almost every animal species and every cell or tissue which has been studied. Many naturally occurring prostaglandins and chemically synthesised analogues possess high biological potency and show a great diversity of pharmacological effects (for review, Horton, 1979).

The role of arachidonic acid and dihomo- γ -linolenic acid as prostaglandin precursors was discovered independently by two groups of workers (Van Dorp, Beerthuis, Nugteren and Vonkemann, 1964; Bergström, Danielsson, Klenberg and Samuelsson, 1964).

A detailed examination of the mechanism of prostaglandin formation has demonstrated that oxygenation occurs initially at the C-11 position in the precursor acids. Evidence for this mechanism was provided by a series of experiments which are summarised in the following paragraph.

Klenberg and Samuelsson (1965) demonstrated, using tritium labelled 8,11,14-eicosatrienoic acid, that the hydrogen atoms in the C-8, C-11 and C-12 positions in the precursor acid were retained in the same positions in PGE₁ formed by sheep vesicular gland incubations. By using

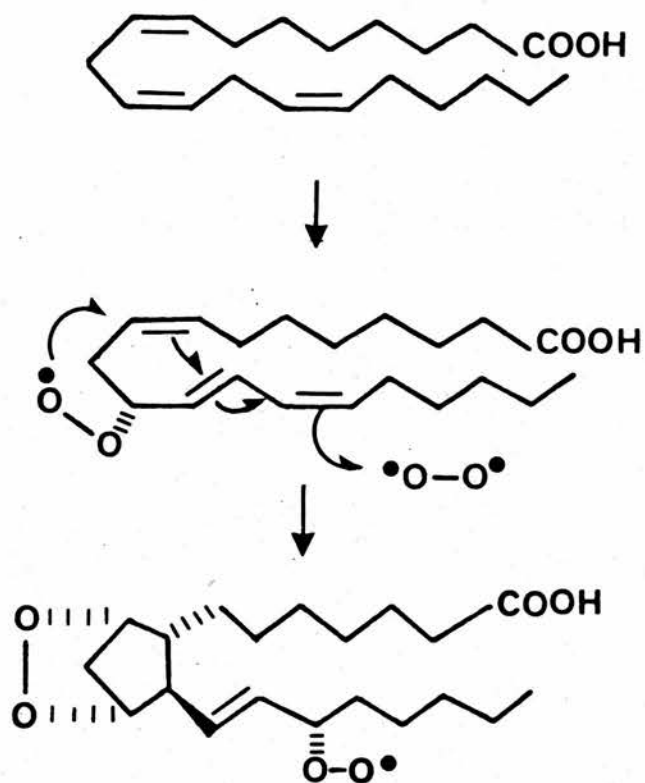


FIGURE 1.2 Mechanism of prostaglandin biosynthesis

$^{18}\text{O}_2$ gas in one biosynthesis of PGE_1 , it was then conclusively demonstrated by mass spectrometric analysis that the oxygen atoms at the C-11 and C-15 positions were derived from molecular oxygen (Nugteren and Van Dorp, 1965; Ryhage and Samuelsson, 1965). Samuelsson (1965) repeated these experiments using a mixture of $^{16}\text{O}_2$ and $^{18}\text{O}_2$ and established that the oxygen atom at C-9 in PGE_1 was derived from one same molecule of oxygen as the oxygen atom in the C-11 hydroxyl group. Further work indicated that the hydrogen lost from C-13 during the biosynthesis of PGE_1 had the L-configuration and that neither 15L-hydroperoxy-8,11,13-eicosatrienoic acid nor 15L-hydroxy-8,11,13-eicosatrienoic acid were converted to PGE_1 by the sheep vesicular gland preparations (Hamberg and Samuelsson, 1967b).

All the evidence indicated that the first intermediary in the biosynthesis of prostaglandins from dihomog γ -linolenic acid was 11-peroxy-8,11,14-eicosatrienoic acid. The initial attack at C-11 was considered to be followed by cyclisation involving attack by oxygen at C-15, isomerisation of the 12,13 double bond into the 13,14 position and formation of an endoperoxide between C-9 and C-11 (Figure 1.2).

In addition to PGE_1 , prostaglandins $\text{F}_{1\alpha}$ and D_1 and 12L-hydroxy-8,10-heptadecadienoic acid (HHD) were also identified as products of the sheep vesicular gland in-

cubations (Hamberg and Samuelsson, 1966; Nugteren, Beerthuis and Van Dorp, 1966; Granström, Lands and Samuelsson, 1968).

Using arachidonic acid as substrate, Hamberg and Samuelsson (1973) and Nugteren and Hazelhof (1973) independently isolated an endoperoxide intermediate (named PGH_2) which contained a hydroxyl group at the C-15 position. An additional endoperoxide with a hydroperoxy group at the C-15 position was also isolated and named PGG_2 (Nugteren and Hazelhof, 1973; Hamberg, Svensson, Wakabayashi and Samuelsson, 1974). In contrast to the previously discovered prostaglandins, the endoperoxides had a short half-life of about five minutes in biological (aqueous) systems.

Miyamoto et al (Miyamoto, Yamamoto and Hayaishi, 1974) resolved two fractions of activity from a microsomal fraction of bovine vesicular glands after solubilisation with Tween 20 and DEAE-cellulose chromatography. One of these fractions formed the unstable endoperoxide(s) when incubated with dihomog- γ -linolenic acid and the second converted the endoperoxide(s) into PGE_1 . These workers demonstrated that indomethacin (10 μM) and aspirin (10 mM) only inhibited the formation of the endoperoxide(s) from dihomog- γ -linolenic acid and not the formation of PGE_1 from the endoperoxide(s).

By 1974, the prostaglandin endoperoxides were known to be precursors to prostaglandins of the A, B, C, D, E and F series and C17 monohydroxy acids (HHD formed from $\text{PGG}_1/\text{PGH}_1$ and 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) formed from $\text{PGG}_2/\text{PGH}_2$). The biosynthetic pathway of PGA and PGB compounds has not been elucidated. However, these compounds can easily be formed from PGE compounds by treatment with base (Anderson, 1969). An enzyme which converts PGA_1 and PGA_2 to PGB_1 and PGB_2 has been identified in plasma from several species. This conversion involves PGC compounds as intermediates (Jones, 1972).

In addition to the prostaglandins and HHT, $\text{PGG}_2/\text{PGH}_2$ were later shown to be precursors of an unstable compound thromboxane A_2 and its stable end product thromboxane B_2 . This pathway was initially elucidated in human platelets and is described in detail in the following subsection. Thromboxane A_2 is a potent inducer of platelet aggregation and a potent vasoconstrictor (Hamberg, Svensson and Samuelsson, 1975; Needleman, Moncada, Bunting, Vane, Hamberg and Samuelsson, 1976).

More recently, Vane and co-workers (Moncada, Gryglewski, Bunting and Vane, 1976) have described an enzyme in blood vessel walls that transforms the endoperoxides into PGI_2 and its stable end product 6-keto- $\text{PGF}_{1\alpha}$. PGI_2 is an unstable compound possessing essentially opposing effects to thromboxane A_2 as it is a potent inhibitor of platelet

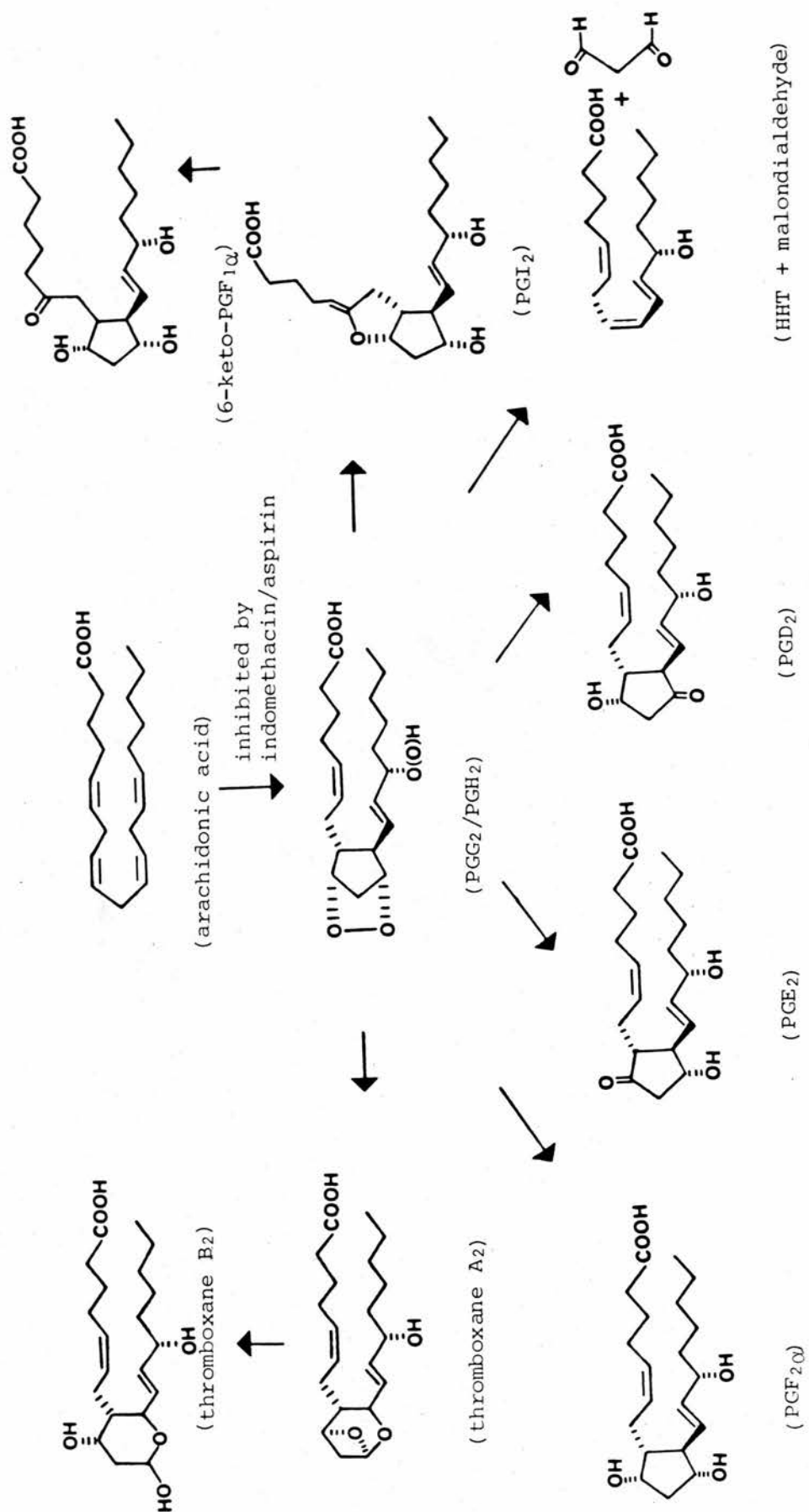


Figure 1.3 Principal pathways of prostaglandin/thromboxane biosynthesis

aggregation and relaxant of smooth muscle (Moncada, Gryglewski, Bunting and Vane, 1976a). The products of this pathway and the implications of the opposing activities of PGI₂ and thromboxane A₂ are discussed in more detail in the following sub-section.

Oxygenation at the C-11 position results principally in the formation of prostaglandins and thromboxanes. The principal pathways involved in prostaglandin/thromboxane biosynthesis are outlined in Figure 1.3 which refers specifically to the products of arachidonic acid. Analogous series of prostaglandins/thromboxanes lacking the 5,6 double bond or having an additional 17,18 double bond are formed if dihomo- γ -linolenic acid or all cis-5,8,11,14,17-eicosapentaenoic acid are used as substrates, the exception being the lack of any natural prostaglandin derived from dihomo- γ -linolenic acid corresponding to PGI₂.

Relative to the prostaglandins, only very small amounts of 11-hydroxy-5,8,12,14-eicosatetraenoic acid (11-HETE) and 11-hydroxy-8,12,14-eicosatrienoic acid were detected following incubations of the respective precursor acids with sheep vesicular gland preparations (Hamberg and Samuelsson, 1967a; Wlodawer and Samuelsson, 1973). In contrast, if 11,14-eicosadienoic acid was used as substrate for the sheep vesicular gland preparations, then 11-hydroxy-12,14-eicosadienoic acid was obtained in high yields (Nugteren, Beerthuis and Van Dorp, 1967).

Small amounts of 11-HETE were also detected in samples from guinea pig lung homogenates incubated with arachidonic acid (Hamberg and Samuelsson, 1974). Recently, Hubbard et al (Hubbard, Hough, Watson and Oates, 1978) tentatively identified 11-HETE as a product following incubation of arachidonic acid with VX₂ carcinoma tissue and Roberts et al (Roberts, Lewis, Lawson, Sweetman and Oates) tentatively identified 11-HETE as the major product of arachidonic acid from mast cells stimulated with the ionophore A23187. It is interesting to note that Hubbard et al reported that 11-HETE formation was not inhibited by indomethacin. This would imply that indomethacin inhibits the cyclisation reaction in the formation of the prostaglandin endoperoxides from the precursor acids.

Oxygenation at the C-12 Position

A lipoxygenase enzyme was identified in blood platelets which possessed ω 9 specificity (Hamberg and Samuelsson, 1974a; Nugteren, 1975). Arachidonic acid and dihomo- γ -linolenic acid were shown to be the best substrates with 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 12L-hydroxy-8,10,14-eicosatrienoic acid, the respective products. The corresponding hydroperoxy compound (12-HPETE) was identified as an intermediate in the conversion of arachidonic acid to 12-HETE. These studies are described in more detail in Section 1.2.

This type of lipoxygenase activity was reported to be detected in a range of tissues (Nugteren, 1977). The highest conversions were observed in those organs which are known to accumulate platelets such as spleen and lung (also Hamberg and Samuelsson, 1974). It was commented that the smaller conversions observed in other organs were probably also caused by platelets, since platelets are present in blood throughout the microcirculation and in principle, they can be expected to be present in every organ.

The platelet lipoxygenase was not inhibited by indomethacin (300 μ M) or aspirin (500 μ M) in contrast to the platelet prostaglandin cyclo-oxygenase which was strongly inhibited by indomethacin (30 μ M) and aspirin (500 μ M). 5,8,11,14-eicosatetraynoic acid (ETYA) (10 μ M) strongly inhibited both enzymes (Hamberg and Samuelsson, 1974a). Hammarström (1977) has reported that 5,8,11-eicosatriynoic acid is a more selective inhibitor of the platelet lipoxygenase in comparison to the prostaglandin cyclo-oxygenase than ETYA.

12-HETE was reported to be chemotactic for human peripheral blood PMNL (Turner, Tainer and Lynn, 1975) and these same workers have reported similar activity of the 12-hydroxy dihomo- γ -linolenic acid product (Turner and Lynn, 1978). They have proposed that these 12-hydroxy compounds may possess roles as chemoattractants in

inflammatory reactions. 12-HETE is reported to lack any appreciable prostaglandin-like biological activity (McGuire, Kelly, Gorman and Sun, 1978). It is inactive on the gerbil colon, produces a weak biphasic response on the blood pressure of the anaethetised rat and has no significant effect on human platelet aggregation.

Oxygenation at the C-5 Position

The first reports of oxygenation at the C-5 position were made by Borgeat and Samuelsson (1976). Following incubation of arachidonic acid with rabbit peritoneal PMNL they identified 5D-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) as the major metabolite. The corresponding hydroperoxy compound (5-HPETE) was postulated as an intermediate but was not isolated. 5-HETE formation was not blocked by the prostaglandin cyclo-oxygenase inhibitor indomethacin (100 μ M) or the platelet lipoxygenase and prostaglandin cyclo-oxygenase inhibitor ETYA (65 μ M).

Later reports (Borgeat and Samuelsson, 1979 and 1979a) indicated that small amounts of 5D,12D-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12-DHETE) were also formed by rabbit peritoneal PMNL incubates and that both 5-HETE and 5,12-DHETE were formed by human peripheral blood PMNL incubates. Using human peripheral blood PMNL, these workers demonstrated that both products were stimulated by the divalent cation ionophore A23187 whereas a third

product, 15-HETE, was not stimulated. They then identified an unstable 5,6-epoxide intermediate in the formation of 5,12-DHETE from 5-HPETE (Borgeat and Samuelsson, 1979b - referenced within Samuelsson, Borgeat, Hammarström and Murphy, 1979).

Slow reacting substance of anaphylaxis (SRS-A) is an acidic lipid of unknown structure initially characterised by Kellaway and Trethewie (1940). It is formed and released from lung, leukocytes and other tissues during allergic reactions as well as in response to a variety of non-immunological stimuli, the most potent of which is the ionophore A23187. SRS-A has potent contractile activity for bronchial and ileal smooth muscle and is considered to be a major cause of bronchospasm during asthmatic attack in man. The stimulation of 5-HETE and 5,12-DHETE by the ionophore A23187 raised the possibility that this pathway was involved in SRS-A formation and release.

Using mouse mastocytoma calls, workers at the Karolinska Institute (Murphy, Hammarström and Samuelsson, 1979 - referenced within Samuelsson et al, 1979) have recently identified the structure of a slow reacting substance and proposed that it was formed from the previously described unstable 5,6-epoxide intermediate in the formation of 5, 12-DHETE. SRS is reported to be a novel cysteinyl derivative of arachidonic acid with a hydroxyl group at C-5, three conjugated double bonds and one isolated double bond

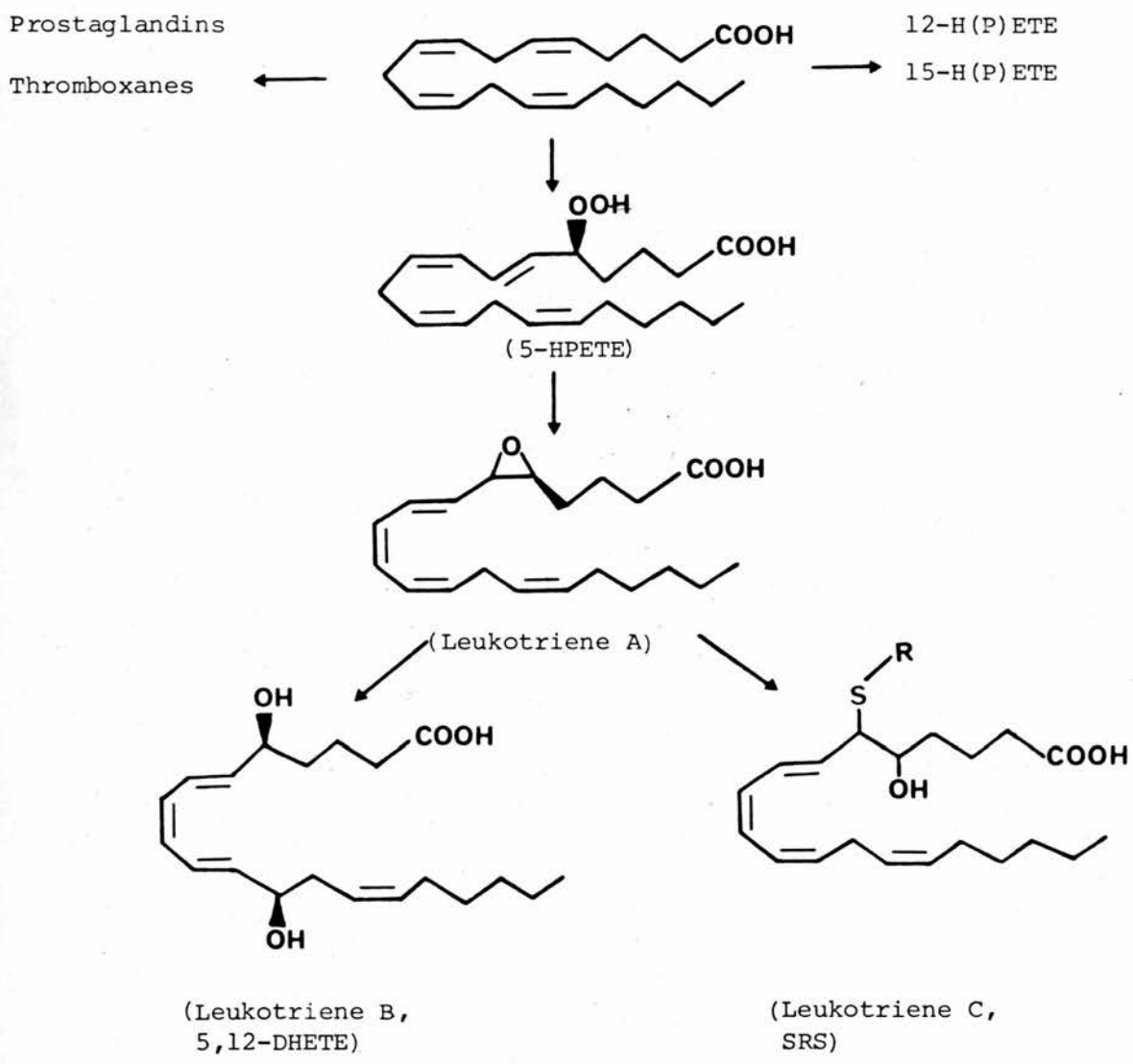


Figure 1.4 Leukotriene nomenclature

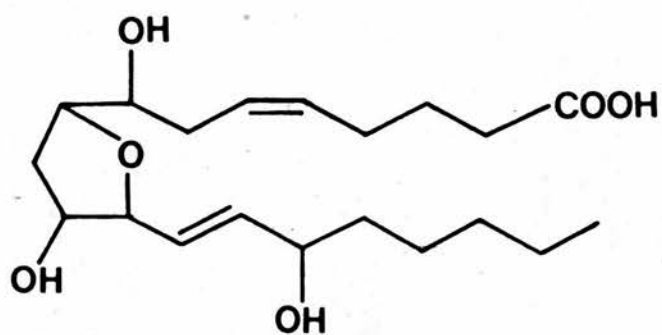
with the amino acid linked as a thioether to C-6. The term leukotriene has been introduced for compounds which like SRS are non-cyclised C20 carboxylic acids with one or two oxygen substitutes and three conjugated double bonds (Figure 1.4).

Recent investigations of the metabolism of arachidonic acid by human peripheral blood lymphocytes (Parker, Stenson, Huber and Kelly, 1979) have suggested that 5-HETE was the major metabolite. Their major product co-migrated with 5-HETE in a number of different TLC solvent systems which adequately resolved 5-HETE, 12-HETE and HHT from each other.

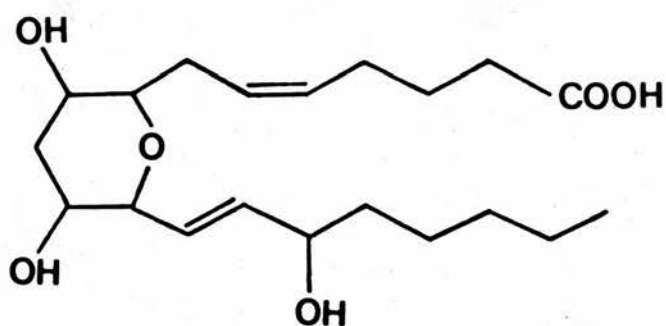
Clearly, this newly identified pathway and its biological implications are an important area for further research.

Oxygenation at the C-8 Position

Incubation of dihomog- γ -linolenic acid with rabbit peritoneal PMNL under similar conditions to those used for the conversion of arachidonic acid to 5-HETE was found to produce 8D-hydroxy-9,11,14-eicosatrienoic acid (Borgeat and Samuelsson, 1976). These workers suggested that it was likely that the formation of 5-HETE and 8D-hydroxy-9,11,14-eicosatrienoic acid were catalysed by two different enzymes but did not rule out the possibility that they were formed by a single lipoxygenase having a higher



Pace-Asciak Compound 1



Pace-Asciak Compound 2

Figure 1.5 Additional metabolites identified by Pace-Asciak

specificity for attack at the C-5 position. Under similar incubation conditions, the formation of 5-HETE was reported to be always favoured over 8D-hydroxy-9,11,14-eicosatrienoic acid.

Pace-Asciak (1971) reported the formation of small amounts of novel arachidonic acid metabolites in addition to previously identified prostaglandins from sheep vesicular gland incubations (Figure 1.5). The analogous compound 1 was also identified from dihomog- γ -linolenic acid incubations. It is possible that formation of one or both of these compounds may involve attack at the C-8 position. No indication was provided as to whether or not these compounds were products of the prostaglandin cyclooxygenase pathway as the effect of indomethacin on their formation was not determined.

Bild et al (Bild, Bhat, Ramadoss and Axelrod, 1978) have isolated high yields (~40%) of a similar compound to Pace-Asciak compound 1 and the analogous dihomog- γ -linolenic acid compound following incubation of an isoenzyme of soyabean lipoxygenase (lipoxygenase-2) with arachidonic acid and dihomog- γ -linolenic acid respectively and subsequent sodium dithionite treatment. This recently described method of preparation of these metabolites is a feasible route for obtaining sufficient material for their biological testing.

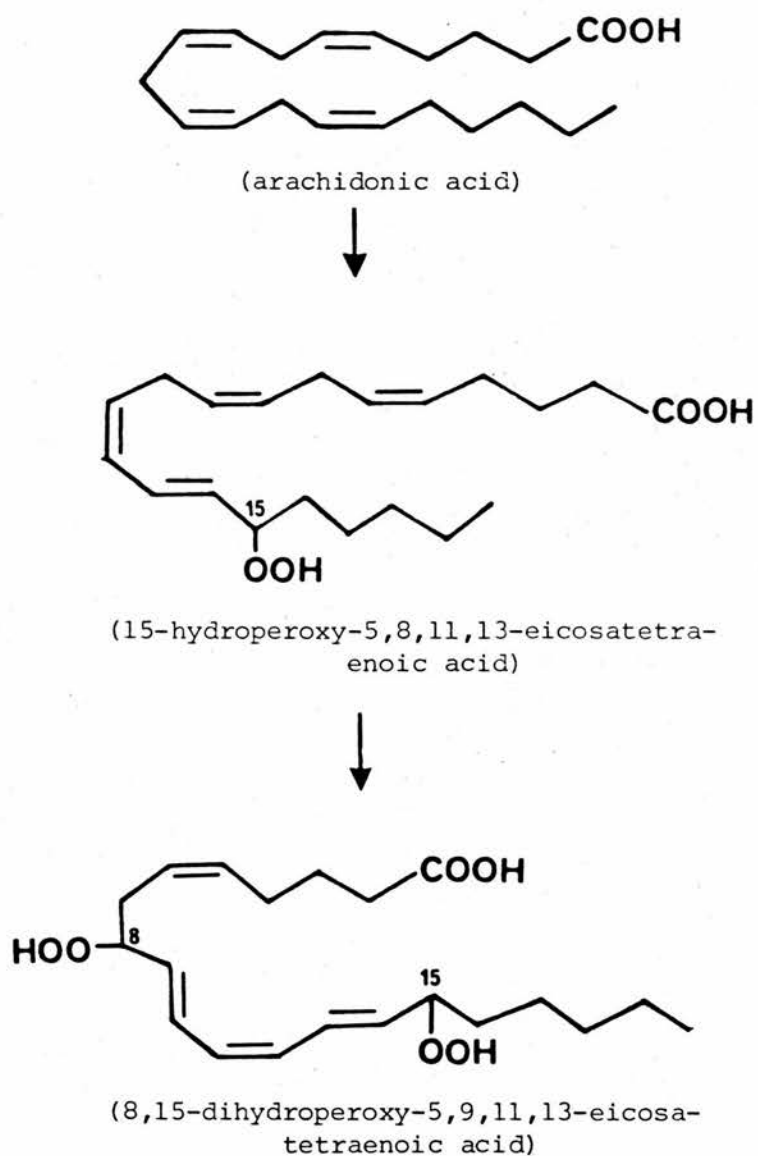


Figure 1.6 Transformation of arachidonic acid by the double-dioxygenation reaction of soyabean lipoxygenase-1

These same workers (Bild, Ramadoss, Lim and Axelrod, 1977) have reported that under high enzyme concentrations of soyabean lipoxygenase-1, arachidonic acid can be oxygenated at two sites on the same molecule to produce an 8,15-di-hydroperoxide containing a conjugated triene according to the reaction scheme in Figure 1.6.

This molecule is reported to inhibit platelet aggregation induced by adenosine diphosphate (ADP), adrenaline and collagen (Bild, Bhat and Axelrod, 1978).

Concluding Remarks

The enzymic oxygenation of both arachidonic acid and di-homo- γ -linolenic acid leads to a wide range of products which possess diverse and at times opposing biological effects. Recent evidence has prompted several researchers to suggest that the more important biological actions of these products are exerted by the unstable arachidonic acid pathway intermediates, the endoperoxides, thromboxane A_2 and PGI_2 . The very recent investigations of Samuelsson and co-workers concerning SRS have opened up another very important area for further research.

The importance of these substances in physiology and medicine and the challenge they pose to synthetic chemistry place them in the forefront of chemical, biochemical and pharmacological investigations.

1.2 THE ROLE OF ARACHIDONIC ACID METABOLITES

IN PLATELET FUNCTIONS

Introduction

The platelet is the anucleate derivative of the largest cell in the bone marrow, the megakaryocyte. Platelet functions include maintaining the integrity of the vasculature, promoting coagulation and responding to blood vessel damage.

The formation of a mass of aggregated platelets at an injury site on a blood vessel is one of the early events in the development of a thrombus. Platelets change shape and aggregate to many agents and it seems likely that they have specific receptors on their surface for most of the aggregating agents to which they are exposed in vivo. These include thrombin, ADP, arachidonic acid metabolites, antigen-antibody complexes, serotonin (5-HT), adrenaline, vasopressin, platelet antibodies, viruses and bacteria.

During the aggregation response to many of these agents, platelets undergo the release of their granule contents and form prostaglandin endoperoxides and thromboxanes from stored arachidonic acid. ADP can also be released which augments the aggregation response to the primary stimulus and the endoperoxides and thromboxanes themselves cause aggregation and further release of ADP.

There are at least three mechanisms which may be involved in the aggregation response - ADP release, endoperoxide/thromboxane formation and another independent mechanism (or mechanisms). The other mechanism (or mechanisms) may occur as a primary aggregation response either before or concurrently with ADP release and endoperoxide/thromboxane formation.

The rest of this sub-section traces the discoveries concerning the involvement of arachidonic acid metabolites in platelet functions.

The Initial Indication of the Involvement of Arachidonic Acid Metabolites in Platelet Functions

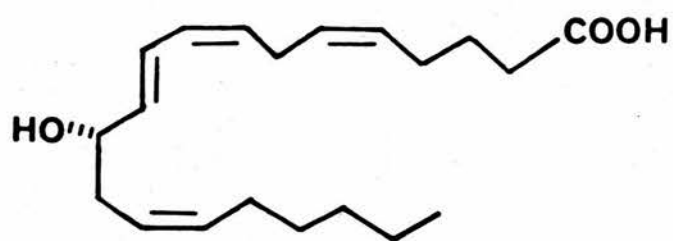
The possible involvement of arachidonic acid metabolites in platelet physiology has been implicated since the late 1960's. Aspirin was reported to prolong bleeding time (Mielke, Kaneshiro, Maher, Weiner and Rapaport, 1969) and to have an inhibitory effect on the second wave of aggregation induced by collagen, adrenaline and ADP (Weiss and Aledort, 1967; Evans, Mustard and Packham, 1967; Morris, 1967; O'Brien, 1968). It was not however until 1971 that it was discovered that aspirin and indomethacin inhibited prostaglandin formation and raised the possibility that the observed effects of aspirin on platelets may be linked with prostaglandin formation (Smith and Willis, 1971; Vane 1971).

The first indication of the formation and release of prostaglandin cyclo-oxygenase products during aggregation was the identification of PGE₂ and PGF₂_α following aggregation induced by various agents (Smith and Willis, 1970; Smith, Ingberman, Kocsis and Silver, 1973). The biosynthetic capacity of PGE₂ by platelets was also demonstrated by Clausen and Srivastava (1971) and Schoene and Iacono (1974). It is worth noting, however, that the latter two groups of workers used PGE co-factors in their incubations.

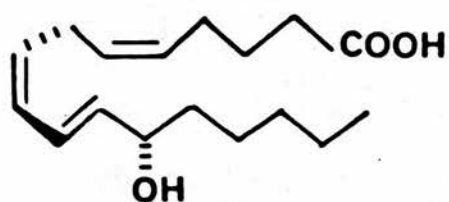
The first evidence that products formed during or as a result of arachidonic acid metabolism via the prostaglandin cyclo-oxygenase pathway mediated shape change, aggregation and the release reaction was the observation that arachidonic acid (in the order of 0.5 mM) induced these factors when added to human platelet rich plasma (PRP) and its effects were abolished by indomethacin or aspirin (Silver, Smith, Ingberman and Kocsis, 1973; Vargaftig and Zirinis, 1973; Smith, Ingberman, Kocsis and Silver, 1974). A number of other fatty acids with similar chain length and degree of unsaturation as arachidonic acid did not cause aggregation of human PRP when tested in millimolar concentrations (Silver et al, 1973).

Detailed Investigations of the Role of Arachidonic Acid Metabolites in Platelet Functions

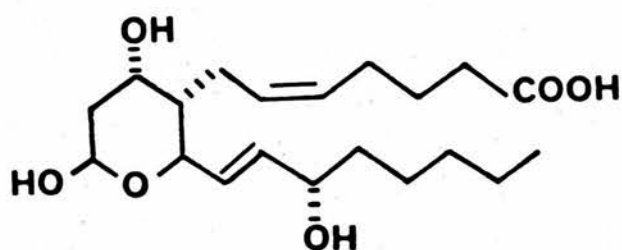
An aggregating material (initially called labile aggre-



(12-HETE)



(HHT)



(Thromboxane B₂)

FIGURE 1.7 Major products identified following incubation of arachidonic acid with washed platelet suspensions

gating stimulating substance, LASS) was reported to be formed from arachidonic acid when it was incubated with preparations of sheep vesicular glands. Extensive studies indicated that the action of LASS was due to the prostaglandin endoperoxides (Willis, 1974; Willis, Vane, Kuhn, Scott and Petrin, 1974).

A series of publications from the Karolinska Institute in Sweden and Unilever Laboratories followed. These elucidated the principal pathways of arachidonic acid transformation in platelets and revolutionised the concepts concerning the role of arachidonic acid metabolites in platelet functions.

The first investigations by the Swedish workers involved the elucidation of the transformation of arachidonic acid by washed platelet suspensions (Hamberg and Samuelsson, 1974a). They identified three major products: 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid (later designated thromboxane B₂) (Figure 1.7). It was demonstrated that only the latter two compounds arose from pathways involving the prostaglandin cyclo-oxygenase pathway, as their formation was inhibited by indomethacin (30 μ M), aspirin (500 μ M) and ETYA (10 μ M). The former product was shown to be formed by a novel lipxygenase (Hamberg and Samuelsson, 1974a; Nugteren, 1975) which

involved the corresponding 12-hydroperoxy compound (12-HPETE) as an intermediate. This intermediate was only isolated in experiments using homogenised platelets and semi-purified enzyme preparations. The lipoxygenase was not inhibited by aspirin (500 μM) or indomethacin (300 μM) but was inhibited by the tetraynoic acid ETYA (10 μM). These results clearly indicated that transformation of arachidonic acid to non-prostaglandin metabolites by platelets occurred to a far greater extent than prostaglandin formation.

These observations, in their turn, prompted a more detailed study on the effects of the endoperoxides on platelet aggregation. They revealed the presence of a new metabolite which was an unstable intermediate in the formation of thromboxane B_2 from the endoperoxides (Hamborg, Svensson and Samuelsson, 1975). The intermediate, named thromboxane A_2 ($t_{1/2}$ 32 sec, 37°C), was trapped by addition of methanol, ethanol or sodium azide to suspensions of washed human platelets incubated for 30 sec with arachidonic acid or PGG_2 . GC-MS examination of the structures of the resulting derivatives demonstrated that the intermediate possessed an oxane ring as in thromboxane B_2 but lacked its hemiacetal hydroxyl group. Additional experiments using $^{18}\text{O}_2$ or $[^2\text{H}]_8$ -arachidonic acid in the formation of thromboxane B_2 and $\text{CH}_3\text{O}^2\text{H}$ for the trapping of thromboxane A_2 , together with information of the half-life of the intermediate indicated its most

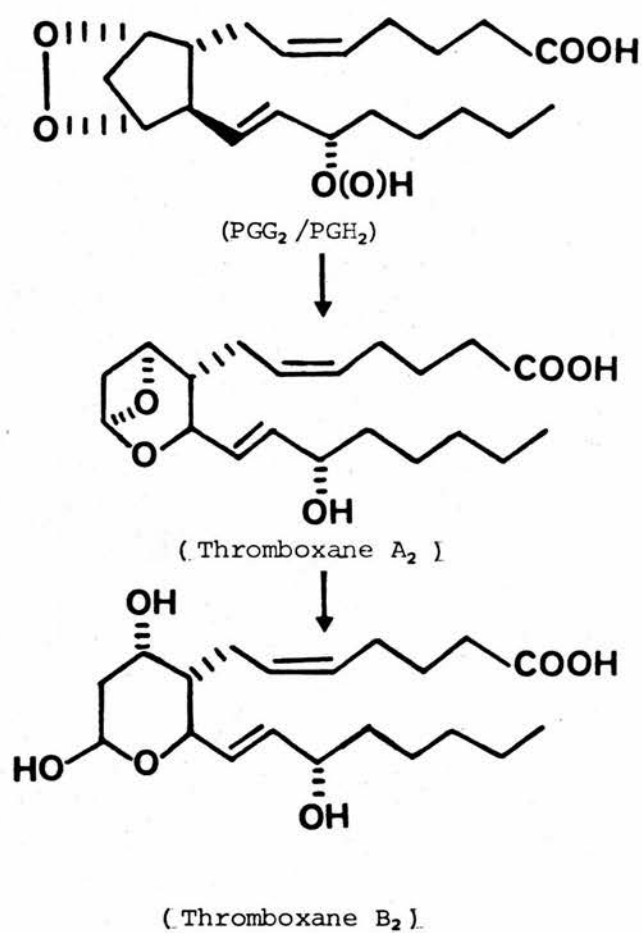


Figure 1.8 Transformation of endoperoxides into thromboxanes

likely structure (Figure 1.8). Thromboxane A_2 was shown to be more potent than the endoperoxides in its ability to induce irreversible aggregation in human platelets and to contract the rabbit aortic strip (Hamberg, Svensson and Samuelsson, 1975; Needleman et al, 1976).

Additional studies demonstrated that, in addition to arachidonic acid, platelet aggregation induced by thrombin, collagen, ADP and adrenaline was accompanied by release of large amounts of thromboxane B_2 , HHT and 12-HETE. PGE_2 and $PGF_{2\alpha}$ accounted only for a very small part of the transformation via the prostaglandin cyclo-oxygenase pathway (Hamberg, Svensson and Samuelsson, 1974; Samuelsson, 1977).

At present, the relative roles of endoperoxides and thromboxanes as aggregating agents is not clear. Needleman and co-workers (Needleman, Minkes and Raz, 1976; Needleman, Raz, Ferrendelli and Minkes, 1977) have maintained that the conversion of the endoperoxides to thromboxane A_2 is not a pre-requisite for induction of platelet aggregation. Studies by Gorman and co-workers have led to somewhat different conclusions (Gorman, Bundy, Peterson, Sun, Miller and Fitzpatrick, 1977) as they deduced that arachidonic acid or PGH_2 will not induce platelet aggregation unless they are converted to thromboxane A_2 . Consideration of the literature leads to the conclusion that, in all probability, the endoperoxides have an aggregating action which can be dissociated from thromboxane A_2 .

However, the latter compound is considerably more potent and when thromboxane A_2 or its stable end product, thromboxane B_2 , can be detected as a major product of an aggregation response, then thromboxane A_2 can be considered as the principal biologically active substance formed via the prostaglandin cyclo-oxygenase pathway.

Oelz et al (Oelz, Oelz, Knapp, Sweetman and Oates, 1977) reported that PGD_2 was also synthesised by human platelets (in comparable amounts to PGE_2) in response to stimuli by thrombin, collagen and adrenaline. Ali et al (Ali, Cerkus, Zamecnik and McDonald, 1977) also reported that human platelets stimulated with arachidonic acid or collagen synthesised PGD_2 in addition to thromboxane B_2 . They found that the amounts of PGD_2 were relatively small compared to thromboxane B_2 ($< 5\%$) in intact washed platelets and platelets in plasma. PGD_2 formation was reported to increase in frozen/thawed platelet preparations. As PGD_2 is known to be a potent inhibitor of platelet aggregation (Nishizawa, Miller, Gorman and Bundy, 1975; Smith, Silver, Ingberman and Kocsis, 1974), these workers have proposed that the formation of PGD_2 may represent a mechanism of feedback inhibition.

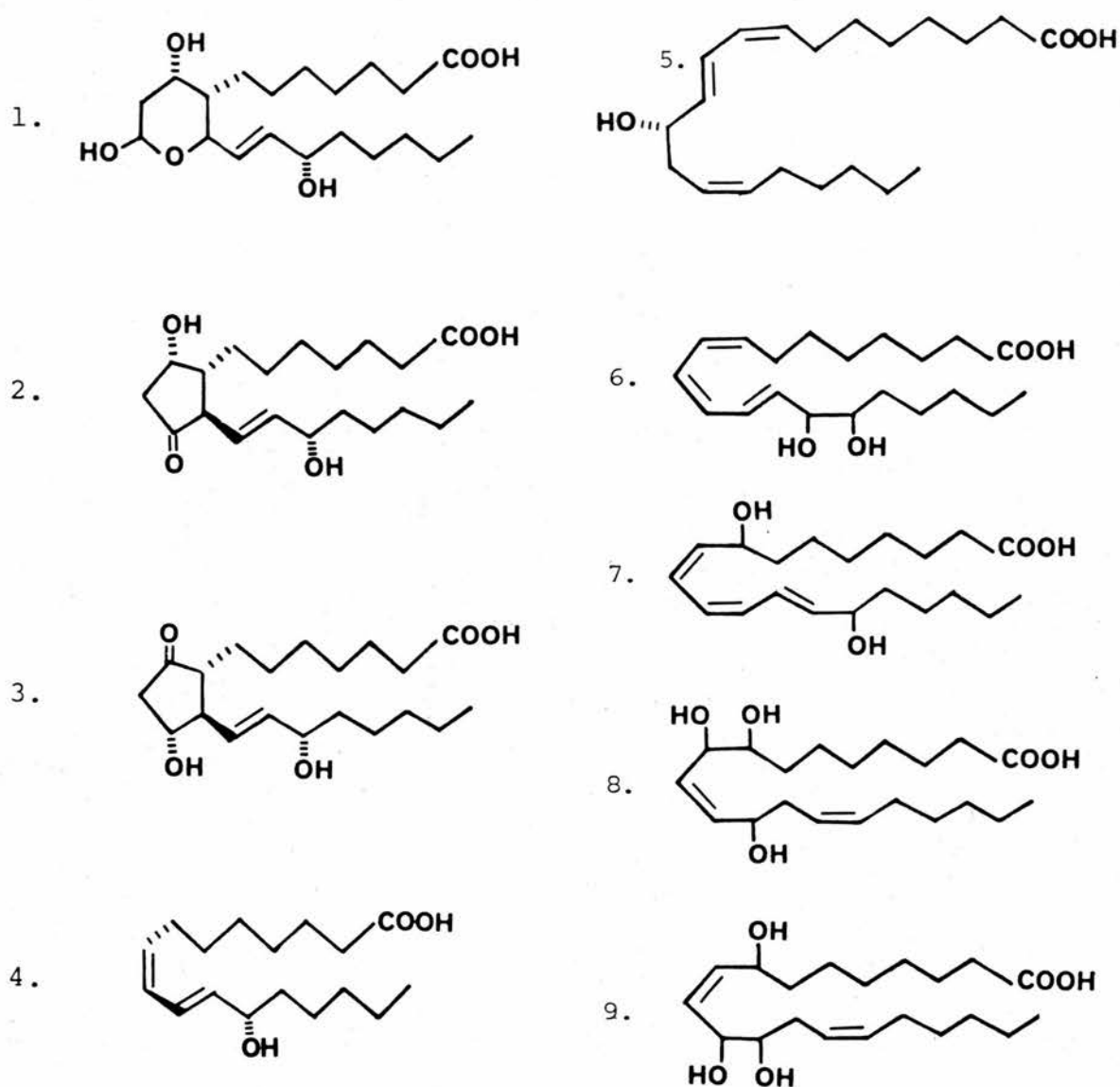
There is no evidence that PGE_2 , $PGF_{2\alpha}$ or PGD_2 are formed enzymatically by platelets and Nugteren (1977) has deduced that they are chemical breakdown products of the unstable endoperoxides because of the very small amounts of these

prostaglandins relative to thromboxane B₂, 12-HETE and HHT. These latter products have been shown to be formed enzymatically.

Characterisation of Enzymes

The platelet lipxygenase enzyme was found in the supernatant of the microsomal fraction of broken platelets. Bovine platelets were used in the study (Nugteren, 1975). This enzyme was shown to have an ω9 specificity and arachidonic acid was the best substrate.

An enzyme was identified in the microsomal fraction of horse and human platelets (Needleman et al, 1976) which converted the endoperoxides (either PGG₂ or PGH₂) into thromboxane A₂. Detailed reports of the biochemical properties of this enzyme (named thromboxane synthetase) from bovine and human platelet microsomes followed (White and Glassman, 1976; Ho, Walters and Sullivan, 1976; Sun, 1977). More recently, Hammarström and Falardeau (1977) and Yoshimoto et al (Yoshimoto, Yamamoto, Okuma and Hayaishi, 1977) have demonstrated the presence of prostaglandin cyclo-oxygenase and thromboxane synthetase in human platelet microsomes. The enzymes have been recovered separately following solubilisation of the microsomes with Triton X-100 and DEAE-cellulose chromatography. These studies (also Hammarström, Lindgren and Roos, 1979) have indicated that the same enzyme is responsible for



- | | | |
|--|---------------------|---------------------|
| 1. thromboxane B ₁ | 2. PGD ₁ | 3. PGE ₁ |
| 4. 12L-hydroxy-8,10-heptadecadienoic acid | | |
| 5. 12L-hydroxy-8,10,14-eicosatrienoic acid | | |
| 6. 14,15-dihydroxy-8,10,12-eicosatrienoic acid | | |
| 7. 8,15-dihydroxy-9,11,13-eicosatrienoic acid | | |
| 8. 8,9,12-trihydroxy-10,14-eicosadienoic acid | | |
| 9. 8,11,12-trihydroxy-9,14-eicosadienoic acid | | |

Figure 1.9 Products identified following incubation of dihomogamma-linolenic acid with washed platelet suspensions

thromboxane A₂ and HHT formation and that HHT is formed directly from the endoperoxides and not from thromboxane A₂. A wide range of inhibitors of this enzyme have been discovered and investigated (Diczfalusy and Hammarström, 1977; Hammarström and Falardeau, 1977). Interestingly, these include the platelet lipoxygenase product, 12-HPETE. Only the hydroperoxy compound had inhibitory action whereas 12-HETE was reported to have no effect.

The Effects of Dihomo- γ -linolenic Acid on Platelet Aggregation

Dihomo- γ -linolenic acid, a polyunsaturated acid closely related to arachidonic acid does not induce human platelet aggregation and furthermore, can inhibit the aggregation caused by various agents (Silver *et al*, 1973; Willis, Comai, Kuhn and Paulsrud, 1974). In order to explain this observation, the metabolism of this acid by washed platelets was investigated by Falardeau *et al* (Falardeau, Hamberg and Samuelsson, 1976). The major product was 12L-hydroxy-8,10,14-eicosatrienoic acid and lesser amounts of 12L-hydroxy-8,10-heptadecadienoic acid, PGE₁, PGD₁, thromboxane B₁; dihydroxy and trihydroxy acids were also detected (Figure 1.9). The structures of two dihydroxy acids were tentatively assigned as 8,15-dihydroxy-9,11,13-eicosatrienoic acid and 14,15-dihydroxy-8,10,12-eicosatrienoic acid - these compounds were resolved by TLC. Structures of two trihydroxy acids which were not resolved, were tentatively assigned as 8,11,12-trihydroxy-9,14-eicosadienoic acid and 8,9,12-trihydroxy-10,14-eicosadienoic acid.

Comparable amounts of thromboxane B₁, PGE₁ and PGD₁ were formed. The endoperoxides derived from dihomo- γ -linolenic acid (PGG₁ and PGH₁) were reported to induce aggregation of washed human platelets although they were less potent than PGG₂ and PGH₂. The lack of aggregatory effect of dihomo- γ -linolenic acid was not therefore explained by the lack of aggregatory activity of the derived endoperoxides but may at least be partly due to insufficient formation of endoperoxides and thromboxanes by the platelets. Furthermore, significant amounts of PGE₁ were formed from dihomo- γ -linolenic acid and PGE₁ is well known to be a potent anti-aggregatory agent (Kloeze, 1967; Kinlough-Rathbone, Packham and Mustard, 1970).

Prostaglandin I₂

Moncada et al (1976) described an enzyme in blood vessel microsomes that transformed the prostaglandin endoperoxides into an unstable substance (initially called PGX and later designated prostacyclin or PGI₂) that relaxed certain blood vessels and inhibited platelet aggregation. PGI₂ was reported to be 20-30 times more potent than PGE₁ and 5-10 times more potent than PGD₂ as an inhibitor of platelet aggregation (Gryglewski, Bunting, Moncada, Flower and Vane, 1976). Prostaglandin I₂ is formed by vascular tissues from all species so far studied and is the main metabolite of arachidonic acid in vascular tissue (for review, Moncada and Vane, 1979).

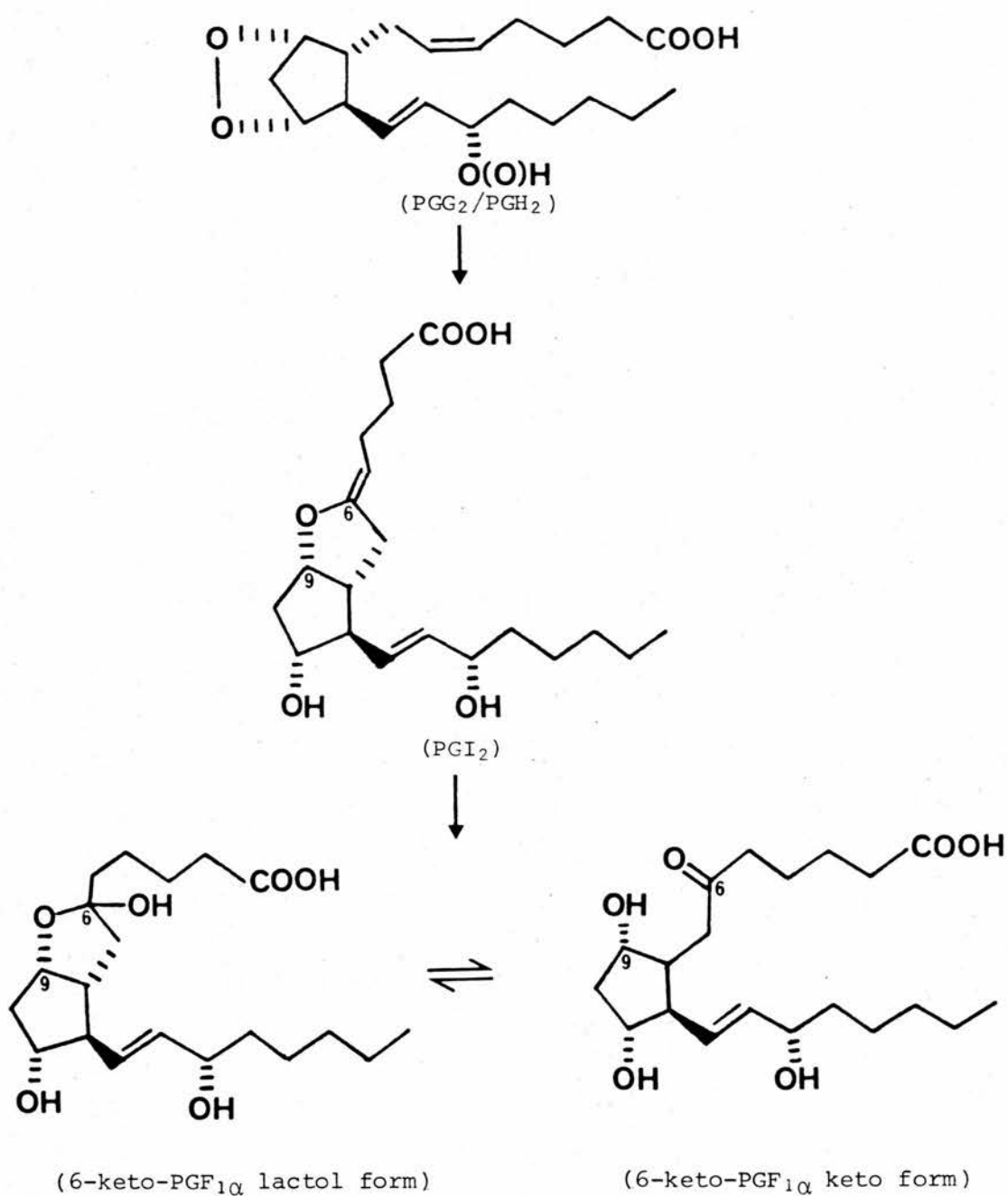


Figure 1.10 Transformation of endoperoxides into PGI_2 and 6-keto- $\text{PGF}_{1\alpha}$

A collaborative study between the Upjohn Company and Vane and his co-workers at the Wellcome Research Laboratories resulted in the elucidation of the structure of PGI_2 (Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada and Vane, 1976). PGI_2 was found to be an unstable enol-ether, intermediate in the formation of the stable 6-keto- $\text{PGF}_{1\alpha}$ (which is in equilibrium with its lactol form) from the prostaglandin endoperoxides (Figure 1.10).

Vane and co-workers have proposed a haemostatic hypothesis whereby the endoperoxides serve as substrates for both thromboxane A_2 and PGI_2 formation - substances with opposing effects. Thromboxane A_2 generated by platelets promotes aggregation while PGI_2 produced by vascular endothelium inhibits aggregation. As corollaries, they have proposed that:

- i) the basal formation of PGI_2 by vascular endothelium may be important in the maintenance of the normal integrity of vessel walls by inhibiting the adherence of platelets.
- ii) PGI_2 may normally limit thrombus formation.
- iii) when a vessel wall is damaged, the formation of a normal haemostatic plug may be assisted by diminished PGI_2 production.

Concluding Remarks

The principal pathways of arachidonic acid metabolism by platelets have been elucidated. The role of the products of the prostaglandin cyclo-oxygenase pathway in platelet functions is considered to be more fully understood than the role of the products of the lipoxygenase pathway. Prostaglandin endoperoxides and thromboxanes are formed and contribute to the aggregation response induced by a number of agents: for example, arachidonic acid, collagen, ADP, adrenaline and thrombin.

Thromboxanes are formed from the endoperoxides which are also precursors for PGI₂, which has opposing biological properties to thromboxane A₂. Several important physiological processes including the haemostatic hypothesis described previously may well be regulated by the opposing biological actions of these two endoperoxide products.

The function of the lipoxygenase pathway has not been elucidated. However, it is interesting to note that two Japanese workers (Okuma and Uchino, 1977) have reported a correlation of platelet aggregability with platelet lipoxygenase deficiency in patients with myeloproliferative diseases and it has been suggested that 12-HETE formation may be part of an elaborate control system for platelet adhesion and aggregation.

The extremely small amounts of prostaglandins and other arachidonic acid or dihomo- γ -linolenic acid metabolites normally present in biological samples necessitates the use of highly sensitive and specific methods for their analysis. Normally, solvent extraction and concentration procedures, followed by purification are applied prior to analysis to increase specificity and sensitivity. The chromatographic mobility of a compound may itself provide part of the evidence of its identity.

The choice of the most suitable chromatographic method for sample purification depends principally on the compounds to be separated, their stability, the mass of the sample and the nature of the sample matrix, and the number of samples to be analysed. Methods used in this field include paper chromatography, thin-layer chromatography, gas and liquid chromatography (including silicic acid chromatography, ion exchange chromatography, reversed-phase partition chromatography, liquid-gel chromatography and high performance liquid chromatography) (Ramwell, Shaw, Clarke, Grostic, Kaiser and Pike, 1971; Horton, 1972; Brash and Jones, 1974; Pryde and Gilbert, 1979).

Biological assay methods have been used extensively for the detection and estimation of prostaglandins and thromboxanes. The advantages are good sensitivity (low nano-

gram amounts) together with specificity for biologically active compounds. Specificity can be enhanced by prior chromatographic purification, by the use of pharmacological antagonists of compounds other than prostaglandins/thromboxanes and by assaying the sample on more than one type of tissue preparation (parallel bioassay). The use of bioassay enabled Samuelsson and his co-workers to detect the highly labile ($t_{1/2}$ 32 sec, 37°C) pro-aggregatory substance thromboxane A_2 (Hamberg, Svensson and Samuelsson, 1975) and Vane and his co-workers (Moncada, Gryglewski, Bunting and Vane, 1976) to detect the labile (activity lost with 10 min at 37°C at pH 7.5) anti-aggregatory substance PGI_2 . The latter example involved an elegant demonstration of using a series of different tissues and detecting a material which produced a spectrum of activity different from all known compounds. The biological significance of these compounds would not have been appreciated without the use of these biological methods of analysis. The obvious disadvantage of bioassay is that inactive metabolites will not be detected.

Radioimmunoassay (RIA) offers good specificity and is capable of measuring metabolites in the nanogram and picogram range. This method can be applied to any metabolite provided that approximately 10 mg of the pure compound is available for the initial raising of the antibody. The unavailability of standards is a limitation to the use of

RIA for the analysis of certain metabolites. A disadvantage is that the technique is time-consuming to set up but this disadvantage can be compensated for by the speed of analysis available with the established technique. Cross-reactivity may cause problems when analysing structurally similar compounds (for example, prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$). These difficulties are usually overcome by prior chromatographic separation of the interfering compounds. The specificity of an RIA method must be thoroughly confirmed by cross-reactivity, accuracy and precision tests as the technique will not provide evidence of interference in any individual sample. In contrast, when estimations are made by combined gas chromatography-mass spectrometry (the reader is referred later in this section), the analyst can observe a characteristically shaped ion current response and scrutinise each individual chromatogram for interfering substances. Ideally, the accuracy of RIA results should be confirmed by another analytical method, preferably gas chromatography-mass spectrometry.

Infra-red and NMR spectroscopy have been employed for the structural elucidation of arachidonic acid and dihomogamma-linolenic acid metabolites (Bergström, Ryhage, Samuelsson and Sjövall, 1963; Anggard and Samuelsson, 1964; Granström, 1971, 1972; Granström and Samuelsson, 1971, 1971a, 1972; Hamberg and Samuelsson, 1974a; Borgeat and Samuelsson, 1976, 1979). However, milligram quantities and very pure samples are required for such analyses and therefore

these techniques are completely unsuitable for the identification of metabolites from small samples of tissue.

Ultraviolet spectroscopy is useful in certain cases, but again, is too insensitive for most analyses and only provides limited information. The method has been successfully applied to the estimation of PGE, PGA and PGB levels in human seminal plasma (ref Bygdeman and Samuelsson, 1964, 1966; Horton, Jones and Marr, 1973).

Combined gas chromatography-mass spectrometry (GC-MS) has been extensively used for the identification, structural elucidation and quantification of arachidonic acid and dihomog- γ -linolenic acid metabolites. The nature of the various polar functional groups in these metabolites necessitates sample derivatisation prior to gas chromatography in order to achieve thermal stability, volatility and hence good gas chromatographic properties.

The carboxylic acid group is rendered less polar by conversion to an ester, most commonly the methyl ester. A number of derivatives have been employed to protect hydroxyl groups and these include the trimethylsilyl (TMS) ether (Luukkainen, Vanden-heuvel, Hahti and Horning, 1961); acetate (Bergström et al, 1963); the boronate ester (Brooks and Watson, 1967) and the tertiary-butyl dimethylsilyl (t-BDMS) ether (Corey and Venkateswarlu, 1972). A free ketone in the ω -side chain of a prostaglandin or in

the PGA or PGB cyclopentane ring need not be derivatised. However, the 1,3 ketol moiety of the E series prostaglandins is particularly labile and must be stabilised by conversion of the ketone to an oxime (Vane and Horning, 1969; Green, 1969).

In conventional gas chromatography (GC), the column eluant is usually monitored by flame ionisation detection (FID) or electron capture detection (ECD). The limit of detection using FID and ECD is in the order of 50 ng and 100 pg respectively. However, in most biological samples there is a large excess of non-precursor acid derived components which cause chromatographic interference and it is this lack of specificity which precludes the use of conventional GC in the analysis of metabolites. These problems of insufficient specificity can be improved by the simultaneous monitoring of mass and radioactivity, a method which has proved useful in metabolism studies (Hamberg and Samuelsson, 1971; Granström, 1972), but only satisfactorily overcome by employing a mass spectrometer as a GC detector (GC-MS).

In a mass spectrometer, a volatilised compound is subjected to ionisation and fragmentation. The individual positively charged ion fragments are resolved in the analyser unit and recorded as a mass to charge ratio (m^+/e value, predominantly charge, $e = 1$). A mass spectrum may be likened to a fingerprint as it is characteristic of the

original molecule. This spectrum combined with the chromatographic retention time of the derivative provides a highly specific method of identification. The gas chromatograph is interfaced to the mass spectrometer by means of a separator, the purpose of which is to remove the GC carrier gas from the analytical sample as it enters the mass spectrometer. These two instruments are highly compatible in terms of quantities of material analysed, response time and requirement for sample volatility. Compounds can be identified by comparison of their chromatographic retention time and mass spectra with those of reference compounds. The structure of new metabolites can be determined from an examination of the chromatographic properties and mass spectra of a variety of derivatives. The following references are excellent examples of this application (ref. Granström and Samuelsson, 1971, 1971a; Granström, 1972; Hamberg and Samuelsson, 1974a; Johnson et al, 1976; Fenwick, Jones, Naylor, Poyser and Wilson, 1977; Borgeat and Samuelsson, 1976, 1979a). Chemical modification or degradation of an identified material followed by GC-MS examination of the products can provide additional useful structural information.

The principal information obtained from a comparison of the most frequently used derivatives can be summarised as follows. In order to determine which of the ion fragments in the methyl ester spectrum of an unidentified metabolite contain the ester group, the spectra of methyl and ethyl

esters are compared. All the ion fragments which are fourteen mass units higher in the ethyl ester spectrum will contain the ester moiety. The number of double bonds may be determined by hydrogenation of the material. On most occasions, the resultant spectrum will be simpler to interpret than the original spectrum and hence provide additional information. The presence (or absence) of a ketone group can be determined depending on whether or not the molecule will form an oxime and similarly, the presence of a 1,2 or a 1,3 diol confirmed by the formation of a boronate derivative. By comparison of spectra of different oximes (or boronates), it can be determined whether or not a particular ion fragment contains the oxime (or boronate) moiety which aids the interpretation of these spectra. These GC-MS procedures in conjunction with other analytical techniques can enable the structure of low microgram quantities of a compound to be determined.

In addition to the use of GC-MS to obtain a fingerprint mass spectrum, it can be used in the selected ion monitoring (also called multiple ion detection) mode. The mass spectrometer is set to monitor only ion fragments of interest. Usually, these are high relative abundance ion fragments in the mass spectrum of the compound to be detected. Throughout a GC run, the ions are focussed alternately on the detector and their intensities are recorded separately as single ion chromatograms. This

method of detection offers much higher sensitivity than recording the whole spectrum of ions and an additional advantage is that individual components may be monitored over the complete time of elution of a GC peak as distinct from a few milliseconds per ion when a mass spectrum is recorded.

In the selected ion monitoring mode, the mass spectrometer can be used both qualitatively and quantitatively. Qualitatively, it is useful to identify smaller amounts of material than would be detected by recording a complete mass spectrum and also to determine whether or not different ion fragments possess the same single ion chromatographic profile. Such information can indicate whether or not the different ion fragments being monitored are derived from the same compound. It may also be useful to determine whether a single ion chromatographic profile is identical to the total ion chromatographic profile. This approach has been applied in Sections 3 and 4 of this thesis.

Quantitative GC-MS employs monitoring the response of the sample relative to an internal reference standard and comparing the response to a set of analytical calibration standards containing the same fixed amount of internal reference standard. The reference internal standard chosen is usually an analogue of the compound to be analysed and ideally is a stable isotope of the compound.

These stable isotopes are normally formed by replacing selected hydrogen atoms by deuterium in various positions of the molecule (for prostaglandins, d_4 compounds containing two deuteriums on C-3 and C-4 are normally used). A fixed amount of internal reference standard is added to each sample at the earliest possible stage in the analytical sequence, usually before the first extraction step and can therefore compensate for varying efficiencies during extraction, purification and derivatisation. A stable isotope internal reference standard and unlabelled standard compound will have virtually identical chromatographic characteristics and will not be distinguished until final detection by the mass spectrometer. At this stage, the instrument is set to record the selected ion chromatograms at the corresponding m^+/e value of the labelled and unlabelled compounds. A calibration curve may then be prepared by plotting the peak response ratio of the standard/internal reference standard against the amount of standard in the calibration standards and a comparison of the peak height ratio of samples enables their quantification. This provides the most specific assay method for the quantitative estimation of small amounts of lipids present in complex biological mixtures. The sensitivity of the method depends on the cleanliness and nature of the derivatised sample - generally low nanograms and on occasions picograms of material can be assayed.

The application of combined gas chromatography-mass spectrometry in order to provide qualitative and quantitative information on metabolites make it an invaluable tool. It is unique in its ability to provide structural information on very small amounts of material - essential information in this area of work. However, the sole use of GC-MS is insufficient. It should ideally be used in conjunction with biological assay methods as only bio-assay can detect short-lived intermediates and provide a clue to the biological significance of metabolites.

SECTION 2

MATERIALS AND METHODS

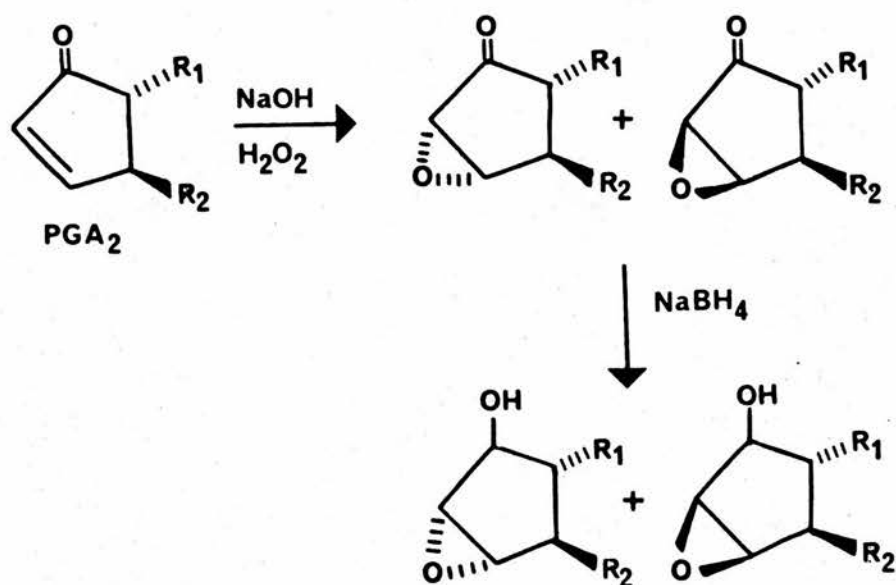


Figure 2.1 Reaction scheme for preparation of $10\alpha,11\alpha$ -epoxy-11-deoxy- $\text{PGF}_2\alpha$ and $10\beta,11\beta$ -epoxy-11-deoxy- $\text{PGF}_2\alpha$

Reference Compounds

Prostaglandins A_2 , B_2 , D_2 , E_2 , $F_2\alpha$, 11,9-epoxymethano- H_2 and thromboxane B_2 were obtained from Dr. J. E. Pike of the Upjohn Company, Kalamazoo, Michigan, U.S.A.

10 α ,11 α -epoxy-11-deoxy-PGF $_2\alpha$ and 10 β ,11 β -epoxy-11-deoxy-PGF $_2\alpha$ were prepared by Dr. R. L. Jones by treatment of PGA $_2$ according to the reaction scheme shown in Figure 2.1. The products of the hydrogen peroxide/sodium hydroxide reaction were subjected to liquid-gel chromatography which resulted in the separation of two isomeric epoxides. These two epoxides were assigned the α and β configurations following GC-MS examination of their products after treatment with aluminium mercury amalgam. One compound yielded a product identical with PGE $_2$ and this epoxide was assigned the α configuration and hence the other was assigned the β configuration. Further treatment of these two isomeric epoxides with sodium borohydride resulted in the two required products whose structure was confirmed by GC-MS. Each product chromatographed as a single zone on LGC, a single spot on TLC and a single peak on GC and it was considered that these products contained an α hydroxyl group.

The methyl ester of oleic acid epoxide was prepared by Dr. N. H. Wilson by treatment of a solution of the methyl

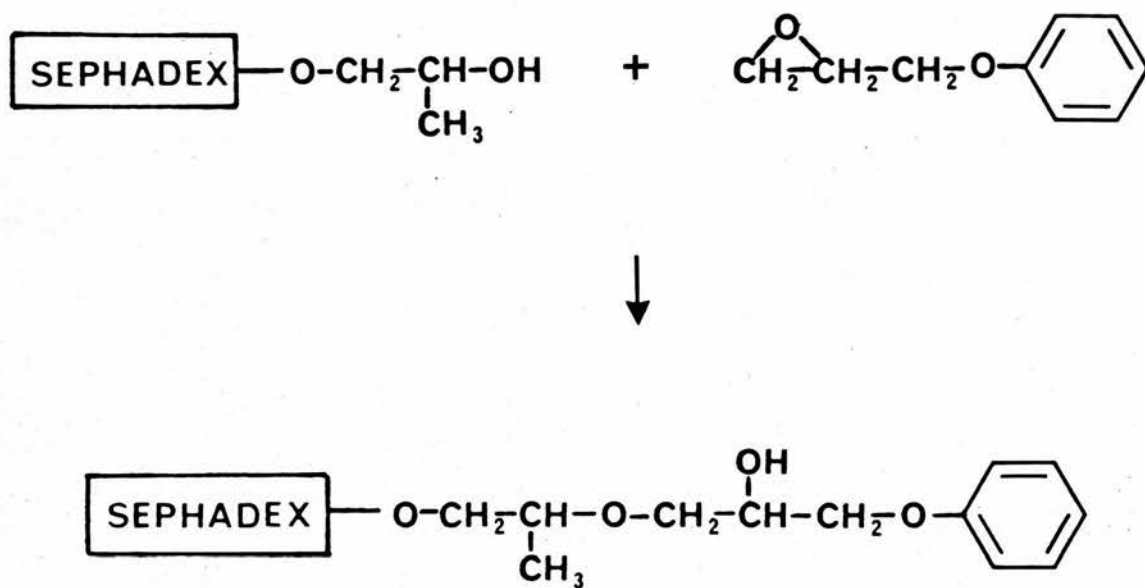


Figure 2.2 Preparation of lipophilic gel

ester of oleic acid in dichloromethane with a slight excess of m-chloroperbenzoic acid for 1 h at 0°C.

Arachidonic acid (99%, Grade 1) was purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. The purity was checked by TLC after each delivery and on one occasion when TLC indicated that there was greater than 10% impurity present, the material was purified by liquid-gel chromatography before use. The arachidonic acid was then prepared and sealed in ampoules under nitrogen as a 10 mg. ml⁻¹ solution in re-distilled ethanol. This storage procedure was shown by TLC to be satisfactory.

1-¹⁴C-Arachidonic acid (60.2 mCi.mmol⁻¹, 197 µCi.mg⁻¹) was purchased from the Radiochemical Centre, Amersham, England and was prepared and stored as a 10 µCi.ml⁻¹ solution in re-distilled ethanol.

Lipophilic Gels

Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Treatment of this gel with 1,2-epoxy-3-phenoxy propane (K & K Laboratories Inc., Hollywood, California, U.S.A.) in dry dichloromethane, with boron trifluoride etherate as catalyst resulted in a product which contained 33% by weight of the phenoxyhydroxypropyl residue (Figure 2.2). This gel had been prepared previously by Drs. R. L. Jones and A. R. Brash.

TABLE 2.1

Balanced salt solutions

Salt solution	Grams per 5 litres			
	Krebs	Tyrode	Earles Salts	Krebs-Henseleit*
NaCl	34.5	40.00	34.00	9.00
KCl	1.75	1.00	2.00	0.46
CaCl ₂	1.40	1.00	1.00	
MgSO ₄ · 7H ₂ O	1.45	1.30	0.50	0.38
NaH ₂ PO ₄ · H ₂ O		0.25	0.62	
Glucose	10.00	5.00	5.00	
NaHCO ₃	10.50	5.00	11.00	
Na ₂ HPO ₄ · 2H ₂ O				2.98
KH ₂ PO ₄	1.60			0.21
Phenol Red			0.25	

*pH adjusted to 7.4 with 1N HCl

Reagents

Analytical grade solvents and inorganic chemicals were used throughout. Toluene, hexane, dichloroethane and isopropanol were HPLC solvents from Rathburn Chemical Co., Walkerburn, Scotland. Pyridine was re-distilled and dried over potassium hydroxide pellets. The suppliers of chromatographic materials and reagents used for derivatisation are given in the appropriate text. The standard salt solutions used in the course of the study are described in Table 2.1.

TABLE 2.2

Composition of washing medium

A.	0.9% (w/v) sodium chloride in distilled water (0.15 M)
B.	Tris(hydroxymethyl)amine/HCl buffer (0.15 M) 9.33 g salt/500 ml distilled water conc HCl added dropwise to pH 7.4
C.	Di-sodium ethylene diaminetetra-acetic acid/NaOH buffer (0.08 M) 14.33 g salt/500 ml distilled water conc NaOH added dropwise to pH 7.4

Washing medium A:B:C
 90:8:2

Preparation of Platelet Rich Plasma

Blood obtained from healthy human volunteers, who had not taken aspirin for 10 days, was collected from the antecubital vein in 0.2 volume of acid citrate dextrose solution (3 g glucose and 2 g disodium hydrogen citrate/120 ml distilled water) in polypropylene centrifuge tubes.

Platelet rich plasma (PRP) was obtained after centrifugation at 200 x g for 15 min at room temperature. The upper plasma layer was aspirated into clean centrifuge bottles.

Blood from dog, cat and rabbit was obtained from animals housed in the Pharmacology Department and was similarly treated (the cat had been treated with indomethacin, Section 2.9.5). The horse blood was obtained from the local slaughter house and was the simplest to handle as the red cells sedimented after standing at room temperature for approximately 30 min following arrival, allowing the PRP to be easily aspirated off.

Preparation of Washed Platelets

Washed platelets were prepared essentially as described by Hamberg et al (Hamberg, Svensson, Wakabayashi and Samuelsson, 1974). This involved the addition of three volumes of washing medium (Table 2.2) to the PRP and centrifugation.

gation at the minimum g force (approximately 600 x g) required to "spin down" the platelets. The supernatant buffer was then removed and the remaining platelet pellet re-suspended in Krebs-Henseleit medium (pH 7.4) which contained neither calcium ions nor glucose. If platelets remained in the supernatant, this procedure was repeated. It was determined that the platelets re-suspended more readily and satisfactorily if they were spun down at their minimum g force in centrifuge bottles with a large bottom surface area as this prevented clumping and aggregation of the platelets.

Three day old platelet concentrate obtained from the Blood Transfusion Service, Edinburgh Royal Infirmary was used on several occasions and was treated in a similar manner. The concentrate was diluted with the washing medium and the small number of red cells present removed by centrifugation at 200 x g for 15 min. The upper layer of platelet rich buffer was aspirated into clean bottles and the platelet pellet was obtained by centrifugation and re-suspended as described above.

When the effects of indomethacin on the products of the platelet incubation were investigated, the PRP was divided into two equal volumes and indomethacin (2 mM solution in ethanol) was added to one volume to give a final concentration of 10 μ M and an equal volume of ethanol was added to the other sample for control purposes. The washed

platelets were then prepared as described previously and the indomethacin again added to the treated sample when the platelets were re-suspended in Krebs-Henseleit buffer.

2.3 INCUBATION AND EXTRACTION CONDITIONS

Incubation Conditions

The washed platelet suspensions (at an approximate concentration of 5×10^8 to $5 \times 10^9 \text{ ml}^{-1}$) were heated to 37°C in a water bath and arachidonic acid was added in a small volume of ethanol and incubated at 37°C . Unless otherwise stated, the final arachidonic acid concentration was $165 \mu\text{M}$ and incubations were carried out for 30 minutes. The final concentration of ethanol was normally 1% (v/v) and never greater than 5% (v/v). $1\text{-}^{14}\text{C}$ -Arachidonic acid (specific activity $120 \mu\text{Ci.mmol}^{-1}$) was added as tracer with the unlabelled arachidonic acid to incubates where the products were to be subjected to liquid-gel chromatography.

When incubations were carried out to determine the effects of indomethacin or ETYA on the products of the incubation, the platelets were pre-incubated for 5 min at 37°C with these inhibitors before the addition of the arachidonic acid. Control samples were prepared and pre-incubated in a similar manner.

In experiments to determine the relative amounts of thromboxane B_2 and THETA produced by washed platelet and PRP incubates, the PRP was divided in two equal volumes and washed platelets were prepared from one volume as described previously. Two volumes of Krebs-Henseleit were

added to the PRP and the washed platelet pellet was re-suspended in the corresponding volume of buffer. Incubations with arachidonic acid ($165\ \mu\text{M}$ in the washed platelet suspension and at three times this concentration in the PRP sample in attempts to compensate for protein binding (Nugteren, 1977; Smith, Ingeman and Silver, 1977)) were carried out at 37°C for 30 min.

After the required time, incubations could be stopped by the addition of five volumes of ethanol to the platelet suspensions. This procedure was adopted in experiments to compare the relative amounts of products after different incubation times, to compare the products from washed platelets and PRP incubates and when the products of washed platelet incubates involving small volumes of buffer were investigated. However, when large volumes were being handled, this procedure was clearly impractical and the addition of ethanol was omitted from the procedure. The incubation mixtures were then centrifuged at $600 \times g$ for 20 min to remove the platelet material and the supernatant retained for extraction. In the case of incubates quenched by the addition of ethanol, the ethanol/water mixture was removed under reduced pressure on a rotary evaporator at 35°C and the residue re-constituted in distilled water prior to extraction.

Extraction Conditions

Following acidification to pH 3.5 - 4.0 with 1N hydro-

chloric acid, the products of the platelet incubation were extracted three times with two volumes of diethyl ether. The combined organic phase was washed once with distilled water (1/10th volume) to remove traces of salts and acids, then taken to dryness under reduced pressure on a rotary evaporator at 30°C.

Non-polar lipids (for example the unreacted arachidonic acid) were routinely removed by partition of the organic extract between 33% aqueous ethanol and petroleum ether (BP 60-80°C) (equal volumes; partitioned twice). Evaporation of the alcoholic phase yielded a residue containing the polar lipids which was sufficiently clean for examination by GC-MS without further purification. This further partition was omitted in samples prepared for the isolation of EPHETA (Sections 4 and 5) as the maximum amount of this compound was required and as this product was not very polar, it was considered possible that a significant amount of the material might partition into the petroleum ether. These samples for the preparation of EPHETA were subjected to liquid-gel chromatography.

Methyl (Me) and ethyl (Et) esters were prepared by treatment of a methanolic solution of the acid with excess diazomethane and diazoethane respectively. The methanol was required to catalyse the reaction (Schlenk and Gellerman, 1960). After five minutes at room temperature the sample was blown to dryness and vacuum desiccated.

Diazomethane was prepared by reaction of ethereal N-methyl-N-nitroso-p-toluene-sulphonamide (Aldrich Chemical Co., Gillingham, England) with aqueous potassium hydroxide and ethanol and transferred into fresh diethyl ether with the aid of a stream of nitrogen. Diazoethane was prepared by reaction of ethereal N-nitrosoethyl urethane (Fluka AG, Buch, Switzerland) with ethanolic potassium hydroxide and transferred into fresh diethyl ether with the aid of a stream of nitrogen and gently warming the reaction mixture in a water bath. Both ethereal solutions could be satisfactorily stored at -20°C for short periods.

Trimethylsilyl ethers (TMS) were prepared by dissolving the esterified material in 30 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). Reaction was carried out for 20 min at 60°C or on several occasions left to proceed overnight at room temperature and the sample then heated on the following morning prior to GC-MS analysis.

Methyl oximes (MO) and butyl oximes (BuO) were normally prepared by treatment of the esterified material with 50 μl of 5 $\text{mg} \cdot \text{ml}^{-1}$ solutions of methoxyamine hydrochloride (Supelco Inc., Bellefonte, Pennsylvania, U.S.A.) and butoxyamine hydrochloride (prepared by Dr. N. H. Wilson in this laboratory) in pyridine respectively. The reaction was left to proceed overnight at room temperature, the sample was heated at 60°C for 40 min on the following morning and then blown to dryness. An alternative method of oximation (Jones, 1972) involved treating the esterified material with a 5 $\text{mg} \cdot \text{ml}^{-1}$ solution of the appropriate amine hydrochloride in 1M sodium acetate buffer (pH 4) containing 20% ethanol in a similar manner. The material was extracted with two volumes of diethyl ether and the extract blown to dryness. Samples were vacuum desiccated and treated with BSTFA prior to GC-MS analysis.

Butyl boronate (n-BuB) derivatives were prepared by treating the esterified material with 50 μl of a 5 $\text{mg} \cdot \text{ml}^{-1}$ solution of n-butane boronic acid (Venton Alfa Products, Beverley, Massachussettes, U.S.A.) in 2,2-dimethoxypropane (Aldrich Chemical Co.) at 60°C for 2 h. The sample was then blown to dryness, vacuum desiccated and treated with BSTFA prior to GC-MS analysis.

Catalytic hydrogenation of the compounds was achieved by bubbling hydrogen through a methanolic solution of the esterified material containing platinum oxide as catalyst,

at room temperature for 2 h. The solution was then filtered through a Whatman No. 50 filter paper (W&R Balston Ltd., Maidstone, England) and the sample blown to dryness and vacuum desiccated prior to further derivatisation and GC-MS analysis.

Reduction of an epoxide with lithium aluminium hydride (LiAlH_4) (Koch-Light Laboratories Ltd., Colnbrook, England) involved dissolving the esterified material in dry tetrahydrofuran in an ampoule. The LiAlH_4 was then added and the ampoule carefully sealed under nitrogen and heated at 60°C for 72 h. After this time, the ampoule was carefully opened and the contents transferred into a small volume of distilled water. The solution was adjusted to pH 4 with 1N hydrochloric acid and then extracted three times with two volumes of diethyl ether. The sample was blown to dryness and vacuum desiccated prior to further derivatisation and GC-MS analysis.

The conditions employed for the ring opening of an epoxide with trimethylchlorosilane and pyridine to form a chlorohydrin-TMS adduct were essentially as described by Harvey et al (Harvey, Johnson and Horning, 1972) and involved heating the esterified material in a mixture of trimethylchlorosilane (Sigma Chemical Co.) ($10\ \mu\text{l}$) and pyridine ($20\ \mu\text{l}$) at 60°C for 30 min. BSTFA ($20\ \mu\text{l}$) was then added in order to silylate any underivatised hydroxyl groups prior to GC-MS analysis.



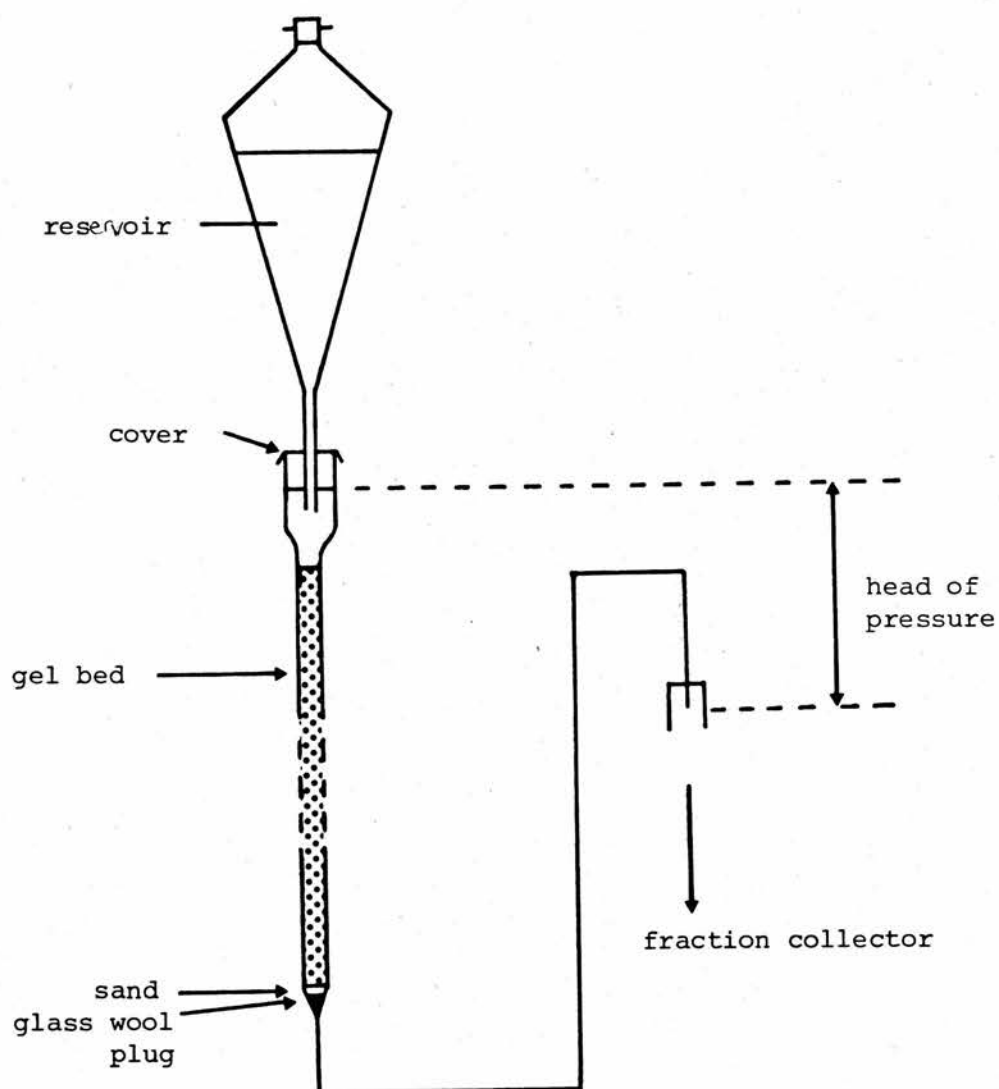


Figure 2.3 Diagram of equipment used for liquid-gel chromatography

Liquid-Gel Chromatography (LGC)

A glass column (45 cm length, 3 cm diameter) was fitted with a constant pressure solvent reservoir. The column was packed with an equilibrated slurry of gel and solvent held in place by a glass wool plug and silanised sand (Figure 2.3) and eluted for at least 12 h before commencement of the first chromatographic separation.

Twice in the course of the study, a decrease in chromatographic resolution made it necessary to clean the gel. This involved unpacking the column and treating the gel with chloroform/methanol/glacial acetic acid (50/50/1). The gel was mixed with this cleaning solvent and the solvent decanted to waste. This procedure was repeated several times. The gel was then re-equilibrated in the required mobile phase and packed as described previously.

Samples were applied to the column dissolved in approximately 2 ml of the mobile phase. An advantage of the toluene based systems used was their good solvent solubility properties to the samples in comparison with hexane/chloroform mixtures which are often used for liquid gel chromatography. The height of the column outflow was adjusted to give a constant flow rate of approximately 25 ml.h^{-1} . Samples were collected on a fixed time basis by the use of a fraction collector (Instrument Specialities

Co., Nebraska, U.S.A.) and those fractions containing the arachidonic acid derived products from the platelet incubates were determined by liquid scintillation counting. A chromatogram was obtained by plotting CPM against fraction number which indicated distinct radioactive zones (Figure 3.6). Fractions from these zones were bulked. When zones were not completely resolved, only the middle fractions from each zone were retained. The eluting solvent was removed from the bulked fractions under reduced pressure on a rotary evaporatory at 35°C, the samples vacuum desiccated and stored in methanol at -20°C prior to analysis. They were analysed as soon as possible after preparation.

Two solvent systems were routinely used:

toluene/1,2-dichloroethane/ethanol (50/50/1) (LGC 1)

toluene/1,2-dichloroethane/ethanol (100/100/5)

+ 0.1% acetic acid (LGC 2).

Thin-Layer Chromatography (TLC)

TLC was carried out using silica gel plates (5 cm x 20 cm, 0.25 mm silica) (Merck AG, Darmstadt, Germany) and silica gel plates with a pre-adsorbent area for facilitated loading (Whatman Inc., Clifton, New Jersey, U.S.A.).

Constant solvent saturation within the tank was achieved by lining the walls with filter paper. Solvent was added

to cover the bottom of the tank, to a depth of approximately 6 mm, and eluted up the filter paper before use.

Arachidonic acid solutions were routinely checked for specified purity by spotting 100 μ g of a methanolic solution onto a plate which was eluted with diethyl ether containing 1% glacial acetic acid. The plate was visualised by spraying with 10% phosphomolybdic acid in ethanol followed by heating at 100°C for 5-10 min. On other occasions, methanolic solutions of material from platelet incubates were applied to the plate and following development, the radioactive zones were detected using a Panax radiochromatogram scanner.

High Pressure Liquid Chromatography (HPLC)

HPLC was performed using a Partisil PXS10/25PAC column (Whatman Inc., Clifton, New Jersey, U.S.A.) fitted with a 50 μ l loop injector (Rheodyne, Berkeley, California, U.S.A) connected to a Dupont 848 Pump (Dupont Instruments, Wilmington, Delaware, U.S.A.). In some experiments, the column eluant was monitored for UV absorbing material using a Cecil CE212 variable wavelength spectrophotometer (Cecil Instruments, Cambridge, England). For PGB₂, the incident light beam was set at 280 nm and for the monohydroxytetraenoic acid 12-HETE at 235 nm. On other occasions when samples contained no chromophore, radioactive tracer was present and fractions of the column

eluant were collected on a fixed time basis using an Ultrorac collector (LKB Ltd., Stockholm, Sweden). Scintillation counting of samples of these fractions indicated the elution time of the radioactive material. On occasions when the chromatographic efficiency of the column had deteriorated, the column was cleaned by eluting with chloroform/methanol (50/50) containing 0.1% acetic acid. After normal use, the column was eluted with methanol for a half-hour period.

Two solvent system were routinely used:

hexane/isopropanol (4/1) (HPLC 1)

hexane/isopropanol (10/1) + 0.1% acetic acid (HPLC 2)

The flow rate was set at 1 ml.min⁻¹.

The GC-MS data obtained in the subsequent sections was obtained using a VG-Micromass 16-16F or a VG-Micromass 70-70F mass spectrometer. Pye 204 gas chromatographs were coupled to each mass spectrometer by means of a single stage glass separator. The gas chromatograph was equipped with a spiral glass column (either 2 m or 3 m x 4 mm internal diameter) packed with 3% OV-1 on Supelcoport 100-120 mesh (Supelco Inc., Bellefonte, Pennsylvania, U.S.A.).

Mixtures of fatty acid methyl esters were used as retention standards. The retention times of the standards were plotted on a logarithmic scale against the number of carbon atoms of the esterified carboxylic acids (Bergström et al, 1963) and the graphs obtained were used to convert observed retention times into carbon values. This fatty acid mixture was also very useful in providing an indication of the chromatographic efficiency of the column and the sensitivity of the mass spectrometer prior to the analysis of samples.

Typical mass spectrometer operating conditions were:

Separator temperature : 250°C

Ion source temperature : 220°C

Electron energy : 22-70 eV (dependent on instrument performance)

Accelerating voltage : 4 kV

Mass spectra were recorded using a light beam oscillograph (S.E. Labs (EMI) Ltd., Feltham, England). Normally, two scans of each peak were recorded at differing amplifications in order to obtain one spectrum with the base peak on scale and, in addition, a second more sensitive spectrum for the detection of low intensity, high mass ion fragments.

The VG-Micromass 70-70F was used in the selected ion monitoring mode with fixed magnet current and voltage scanning. The exact mass measurements of the OV-1 phase column bleed ion fragments (which were initially determined using perfluorokerosene as calibration standard) were used for reference purposes. The mass spectrometer was tuned to the required ion fragment for selected ion monitoring by reference of its precise mass to the precise mass of the nearest column bleed ion fragment and single ion chromatograms were recorded on a multi-pen recorder (Rikadenki, Mitsui Electronics (UK) Ltd.).

All radioactive samples were counted in a refrigerated liquid scintillation counter (Nuclear Chicago Ltd., High Wycombe, England). The scintillation fluid for monitoring the column eluant of the liquid-gel chromatographic separations was a 0.8% solution of 2,5-diphenyl oxazole (Fisons Scintillation Reagents, Loughborough, England). In other instances, 1 volume toluene:2 volumes PCS (Amersham Corporation, Arlington Heights, Illinois, U.S.A) was used.

Samples with the same quenching properties, for example, chromatographic fractions, were compared as counts per minute (CPM). In other cases, the CPM were converted to disintegrations per minute (DPM) by the routine use of the automatic external standard method which allowed the percentage efficiency of each individual sample to be calculated. All quantitative determinations were carried out on the calculated DPM values.

Quantification of metabolites was determined by comparison of the amount of radioactivity in each sample with the initial amount of radioactivity added to incubates (this was equivalent to a known amount of arachidonic acid).

Ultraviolet spectra were recorded with a Pye Unicam SP 800B Spectrophotometer (Pye Unicam Ltd., Cambridge, England) in a 3.5 ml silica glass cell, path length 1 cm. Spectra of solutions of samples were obtained against their solvent in the reference cell.

1. Platelet Aggregation

Platelet rich plasma (PRP) was prepared as described previously (Section 2.2) and kept at room temperature. Platelet aggregation was monitored photometrically at 37°C using a Born Aggregometer (H. Upchurch & Co. Ltd., Leicester, England (Born, 1962) connected to a Servoscribe pen recorder (Belmont Instruments, Glasgow). 0.5 ml of PRP and 0.5 ml of 0.9% (w/v) sodium chloride solution were added to silanised glass tubes, placed in the aggregometer and stirred for three minutes which allowed the platelet suspension to reach the required temperature of 37°C.

The aggregation response, which resulted in an increase in light transmittance, was monitored following the addition of different doses of arachidonic acid or adenosine diphosphate (ADP) and hence a dose was determined for each compound which was slightly greater than the minimum dose required to induce irreversible aggregation. This dose was used in subsequent testing of potential inhibitors. These were added to the platelet suspension in saline or in a small volume of methanol (< 3%, v/v, this volume was shown to have no direct effect on the aggregation

response) and pre-incubated for five minutes at 37°C before the addition of the arachidonic acid or ADP.

After each experiment, the glass tubes were washed, re-silanised using a 5% solution of dimethylchlorosilane in toluene and rinsed with methanol.

2. Prostaglandin I₂ Production

The method of preparation and assay of prostaglandin I₂ was similar to that described by Bunting et al (Bunting, Gryglewski, Moncada and Vane, 1976a). Prostaglandin I₂ was produced by incubating arachidonic acid with aortic rings and monitored, by its powerful anti-aggregating effects on platelet aggregation induced by ADP, using a Born Aggregometer as described in the previous sub-section.

The PRP was treated with indomethacin (10 µM) to negate any potential effects of unreacted arachidonic acid in the aortic ring incubate samples. A dose response curve of the platelets to ADP was prepared and hence a dose slightly greater than the minimum dose required to induce irreversible aggregation was determined. This dose was used in the subsequent determinations.

An abdominal aorta was obtained from a freshly sacrificed rabbit and connective tissue disconnected.

The strip of aorta was weighed and then stored on ice on a piece of cotton wool soaked in tris(hydroxymethyl)-amine-HCl buffer (0.05M, pH 7.5). Aortic rings (approximately 10 mg) were cut from the strip of tissue as required, washed six times with tris buffer and incubated with arachidonic acid (30 μ M) in 0.2 ml tris buffer for three minutes at room temperature. Aliquots of incubates were immediately transferred into the platelet suspension (already at 37°C in the aggregometer) and incubated with the platelets for one minute prior to the addition of ADP. The anti-aggregatory properties of the aortic ring incubates (which were an indication of PGI₂ synthesised) were therefore determined.

A control response of the platelets to ADP was determined after testing a set of three aortic ring incubates in order to check that addition of ADP to the platelets still resulted in a similar aggregation response. If the platelets had become less sensitive then the dose of ADP was increased until a response similar to that obtained previously was attained.

The inhibitory effect of a compound on PGI₂ synthesis was tested by soaking the aortic ring in 0.2 ml of tris buffer containing the compound for 5 min at 0°C, decanting the buffer, adding a further 0.2 ml buffer containing the compound, incubating with arachidonic

TABLE 2.3

Non-prostaglandin antagonists used in superfusion

Drug	Concentration	Antagonist to
Methysergide bimaleate	400 $\mu\text{g.l}^{-1}$	5-HT
Propranolol hydrochloride	3 mg.l^{-1}	Catecholamines (beta effects)
Phenoxybenzamine hydrochloride	100 $\mu\text{g.l}^{-1}$	Catecholamines (alpha effects)
Hyoscine hydrobromide	100 $\mu\text{g.l}^{-1}$	Acetylcholine
Mepyramine maleate	8 mg.l^{-1}	Histamine
Indomethacin	3 mg.l^{-1}	Inhibitor of endogenous PG synthesis

acid and monitoring the PGI_2 produced as described above. Inhibition of synthesis resulted in less "anti-aggregatory activity" in the aortic ring incubate and when this activity was monitored using the platelet system, this resulted in an aggregation response when ADP was added. When the inhibitory effect of indomethacin was tested, an additional amount of indomethacin was added to the ADP control response to compensate for the potential effect of the amount transferred from the aortic ring incubate. This was not necessary for the other compounds tested as they had been shown to have no direct effect on the ADP aggregation response.

3. Tissue Preparations

A rat fundus and rabbit aorta were obtained from freshly sacrificed animals and prepared for bioassay as described previously (Staff, University of Edinburgh). In both cases, the tissues were attached (under tension [3 g fundus; 4 g aorta]) by threads to smooth muscle isometric transducers which were connected to a Grass polygraph multi-channel pen recorder (Grass Instrument Co., Massachusetts, U.S.A.) and superfused at $7.5 \text{ ml} \cdot \text{min}^{-1}$ with Krebs solution at 37°C which contained a mixture of non-prostaglandin antagonists (Table 2.3). This increased the specificity of the assay tissues to

compounds acting on prostaglandin receptors. Both tissues were superfused for approximately 1 h before use. Thereafter, a 10 min dose cycle was adopted when either standard prostaglandins, prostaglandin analogues or test compounds were injected (in Krebs, saline or saline containing a small amount of sodium bicarbonate) onto the tissues.

A guinea pig ileum was used as an assay tissue and was prepared for bioassay as described previously (Staff, University of Edinburgh). It was attached under 0.5 g tension by threads to an isotonic transducer connected to a Grass Polygraph in an 8 ml organ bath in Tyrode. The tissue was washed repeatedly for the first 30 min and thereafter a 4 min dose cycle was adopted when either acetylcholine, a standard prostaglandin or test compound were injected in saline (or saline containing a small amount of sodium bicarbonate) into the organ bath. The compound was allowed a 30 sec contact time, after which the tissue was washed for 1.5 min and left to stabilise for a further 2 min.

4. Chemotaxis

Preparation of Polymorphonuclear Leukocyte (PMNL) Suspensions

Rabbit peritoneal PMNL suspensions were obtained by

the technique of Hirsch and Church (1960). Dutch rabbits (2-3 kg) were used with intervals of 7 to 10 days between leukocyte collections from the same animal. 200 ml of 0.1% (w/v) oyster glycogen (Calbiochem, San Diego, California, U.S.A.) in 0.9% (w/v) sterile sodium chloride solution (saline) was administered by intraperitoneal injection. Four hours later, the peritoneal cavity was washed out with 100 ml TC199 (Gibco-Biocult, Glasgow, Scotland) fortified with Earles salts, 25 mM HEPES buffer (N-2 hydroxyethyl-piperazine-N-ethane sulphonic acid), benzyl penicillin ($100 \text{ units.ml}^{-1}$) and streptomycin ($100 \mu\text{g.ml}^{-1}$). The cell suspension was collected in silanised centrifuge tubes containing heparin (5 units.ml^{-1} final volume).

Differential leukocyte counts showed the PMNL to be the predominant species. Occasionally, PMNL viability (measured by trypan blue exclusion) fell below 95% or the leukocyte suspensions were contaminated with erythrocytes. These suspensions were discarded. Suspensions containing $5 \times 10^6 \text{ PMNL.ml}^{-1}$ were prepared by centrifugation ($52 \times g$ for 10 min) of a known volume containing the appropriate number of cells. The cell pellet was suspended in the correct volume of fortified TC199 containing bovine serum albumin (BSA, $100 \mu\text{g.ml}^{-1}$) (Sigma Chemical Co.).

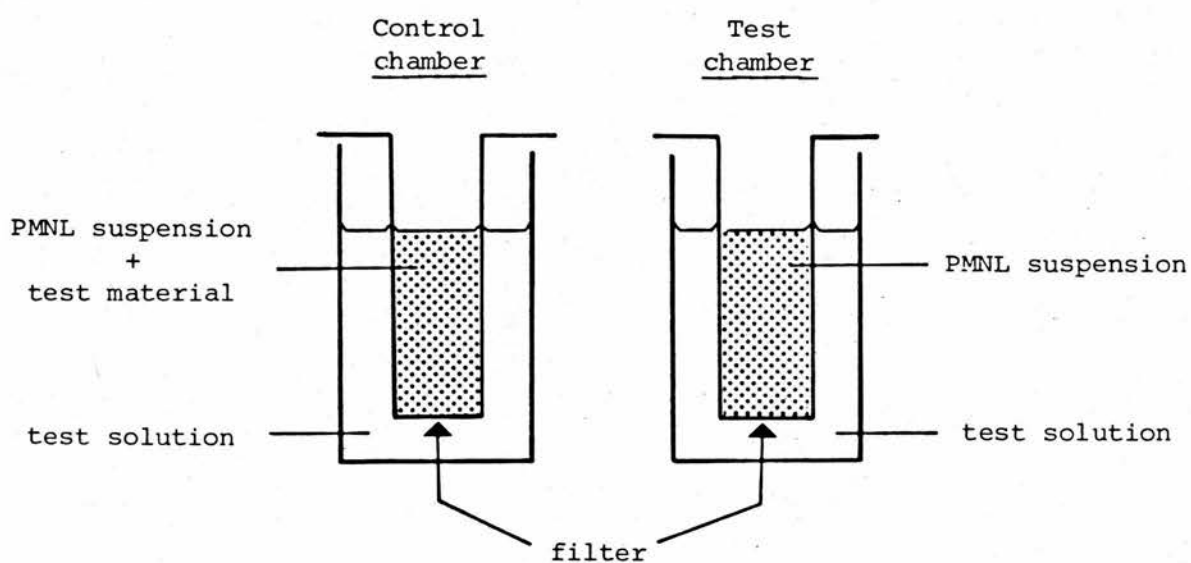


Figure 2.4 Modified Boyden chamber used in chemotaxis experiments

Measurement of Chemotaxis

Disposable polystyrene chemotactic chambers (Adaps Inc., Dedham, Massachusetts, U.S.A.) based on the principle of Boyden (1962) and mixed cellulose acetate/cellulose nitrate filters with 8.0 μm pore size (Membrane SCWP 01300, Millipore S.A., Molsheim, France) were used for the measurement of chemotaxis. The filter was cemented to the upper compartment of the chemotactic chamber (M.F. Cement, Millipore Corp., Bedford, Massachusetts, U.S.A.).

All compounds to be investigated were prepared in fortified TC199 containing BSA ($100 \mu\text{g}.\text{ml}^{-1}$) 2 h before required. Test and control chambers were prepared for each compound. The test chamber contained test solution (1.0 ml) in the lower compartment and 0.9 ml PMNL suspension plus 0.1 ml fortified TC199 (+ BSA) in the upper chamber. Control chambers contained test solution (1.0 ml) in the lower compartment and 0.9 ml PMNL suspension plus 0.1 ml fortified TC199 (+ BSA) containing the same amount of test material in the upper compartment (Figure 2.4). Chambers containing no test materials were prepared for each experiment to provide background values of leukocyte migration. The chambers were incubated for 3 h at 37°C in a humid environment.

TABLE 2.4

Staining procedure for chemotaxis filters

1.	Rinsed with fortified TC199
2.	Methanol for 5 sec
3.	Harris' haematoxylin for 5 min
4.	Rinsed with distilled H ₂ O
5.	Acid alcohol (ethanol + 1% conc HCl) for 1 min
6.	Rinsed with distilled H ₂ O
7.	Blueing agent (2 g sodium bicarbonate + 20 g magnesium sulphate.l ⁻¹) for 20 min
8.	Rinsed with distilled H ₂ O
9.	70% ethanol for 2 min
10.	95% ethanol for 2 min
11.	Absolute ethanol for 2 x 3 min
12.	Xylene

The filter was then removed, stained according to the procedure in Table 2.4 and mounted on a microscope slide with DPX mountant (BDH Chemicals Ltd., Poole, England). The lower surface of the filter was examined microscopically and the mean number of PMNL from five randomly selected high power fields (objective x40) was calculated to give an indication of the chemotactic index.

An increase in cell count in comparison to the background when the compound was present in equal concentration on both sides of the chamber (control chamber) gives an indication of chemokinetic activity. Similarly, an increase in cell count in the test chamber in comparison to the control chamber gives an indication that the substance is exerting a chemotactic influence.

Equilibration studies involved setting up six identical chambers containing $\text{PGF}_2\alpha$ (1 μg) with $^3\text{H-PGF}_2\alpha$ as tracer in the lower part of the chamber and the PMNL suspension in the upper part of the chamber. At regular intervals (1, 2 and 3 h), two chambers were removed from the incubator and 0.5 ml sampled from each side of each of the chambers. The amount of radioactivity in each of the samples was determined by liquid scintillation counting and hence the percentage of radioactivity in each part of the chamber was calculated.

5. Blood Pressure Experiments

Two male cats (weight 2.8 and 3.0 kg) were initially anaesthetised with sodium phenobarbitone (40 mg.kg^{-1}) and a maintenance infusion was made into the internal jugular vein (36 mg.h^{-1}). Blood pressure was recorded from the common carotid artery and the heart rate was recorded on a tachometer triggered from the blood pressure pulse. Drugs were infused intravenously into the femoral vein in saline (or saline containing a small amount of sodium bicarbonate). On one occasion after the cat was killed, its blood was collected and PRP and washed platelets prepared and incubated in the normal manner (Sections 2.2 and 2.3) and the products examined by gas chromatography-mass spectrometry.

SECTION 3

THE INCUBATION OF ARACHIDONIC ACID
WITH WASHED BLOOD PLATELETS WITH THE DETECTION
AND SUBSEQUENT STRUCTURE ELUCIDATION
OF NOVEL METABOLITES

PART I

Thromboxane B₂ was required in the laboratory as a reference standard for a proposed GC-MS investigation of a number of biological samples and was not, at the time, available from the Upjohn Company. Washed blood platelets incubated with arachidonic acid are reported to produce thromboxane B₂ as a major product (Hamberg and Samuelsson, 1974a) and therefore it was decided to attempt the preparation of thromboxane B₂ by this method.

Incubation of arachidonic acid with washed horse platelets and subsequently washed human platelets revealed the presence of novel arachidonic acid metabolites. Purification of the incubation extracts by liquid-gel partition chromatography, followed by a GC-MS examination of a number of derivatives of selected chromatographic zones provided structural evidence for stereoisomers of two trihydroxy acids - 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid (8,11,12-THETA) and 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid (8,9,12-THETA), termed THETA collectively. The structural evidence obtained by GC-MS and the factors affecting the formation of these trihydroxy acids are discussed.

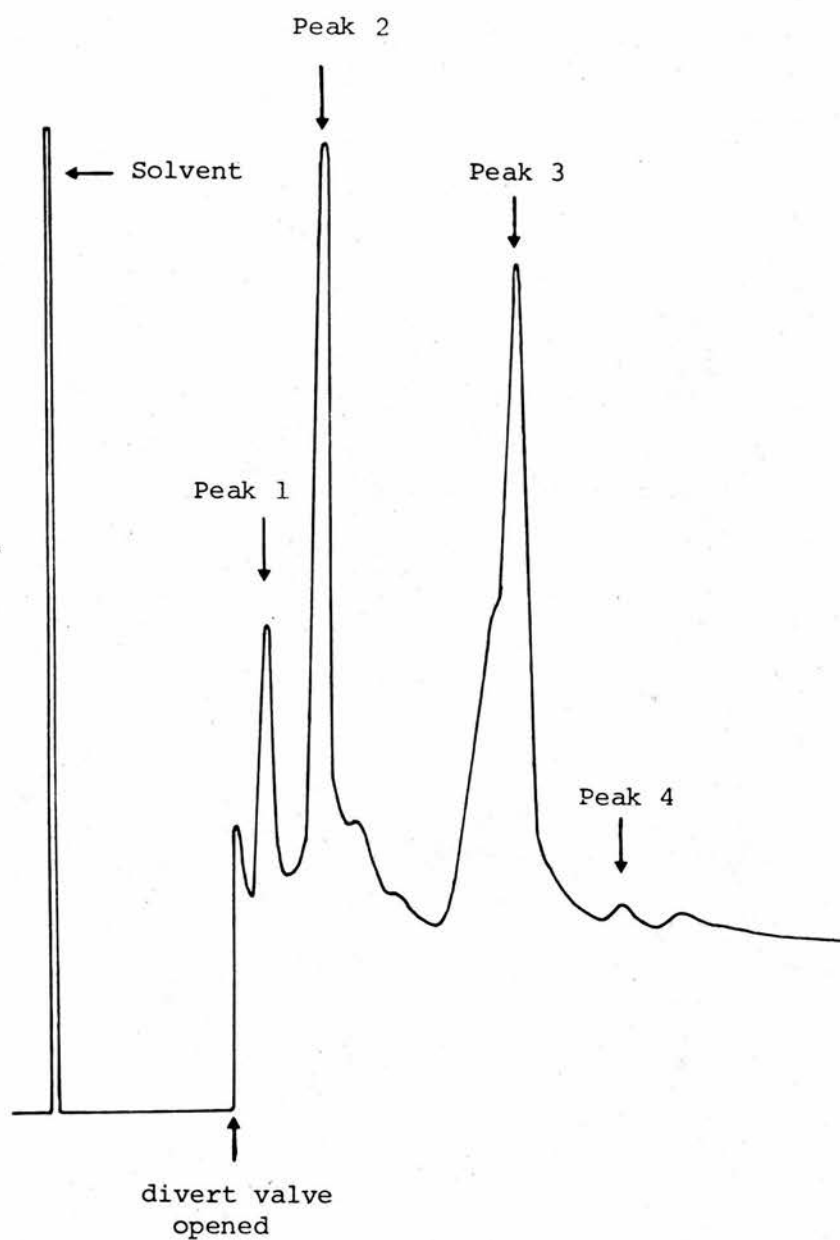


Figure 3.1 Gas chromatogram of 33% aqueous ethanol extract from a washed horse platelet incubate sample (Me/TMS)

3.2 THE INITIAL EXAMINATION OF PLATELET INCUBATES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Examination by GC-MS of the methyl ester/trimethylsilyl ether (Me/TMS) derivatives of the 33% aqueous ethanol extracts from washed horse platelet incubates on three separate occasions indicated that only very small amounts of thromboxane B₂ were present. Peak 4 in the chromatogram (Figure 3.1) was identified as thromboxane B₂ (Me/TMS) by comparison of its retention time (carbon value 24.6) and mass spectrum with published data (Hamberg, Svensson and Samuelsson, 1975; Smith, Harland and Brooks, 1977). It was noted that the horse platelet rich plasma did not aggregate to arachidonic acid.

Incubation of horse platelets with arachidonic acid was clearly not a satisfactory method for the preparation of thromboxane B₂. The very small amounts of thromboxane B₂ identified in the horse platelet incubates together with the observation that the horse platelet rich plasma did not aggregate to arachidonic acid raised the following possibilities:

- a) the horse platelets were deficient in the prostaglandin cyclo-oxygenase enzyme
- b) the horse platelets were deficient in the thromboxane synthetase enzyme

- c) the animals had received drug treatment before their death, or diet which resulted in an inhibition of either the prostaglandin cyclo-oxygenase and/or thromboxane synthetase enzymes

Deficiency of thromboxane synthetase seemed unlikely in view of the fact that horse platelet microsomes have been used for the preparation of thromboxanes from prostaglandin endoperoxides (Needleman et al, 1976) and are known to be good source of thromboxane synthetase. In contrast, there were no previous reports of using horse platelets to prepare thromboxanes from arachidonic acid and in light of the above observations, it seemed a possibility that these platelets might be deficient in the prostaglandin cyclo-oxygenase enzyme. No information was available concerning drug treatment of the animals.

The other GC peaks in the chromatogram of the horse platelet incubate sample were examined. Interest became centred on a major GC peak (peak 3, Figure 3.1) which had a slightly shorter chromatographic retention time (carbon value 23.6 - 23.8) than thromboxane B₂. GC-MS evidence indicated that this same material was formed when washed human platelets were incubated with arachidonic acid. In the case of the human platelet incubates, the relative amounts of thromboxane B₂ and this unidentified material varied considerably. Sometimes, the thromboxane B₂ was present in larger amounts, in others, the unidentified

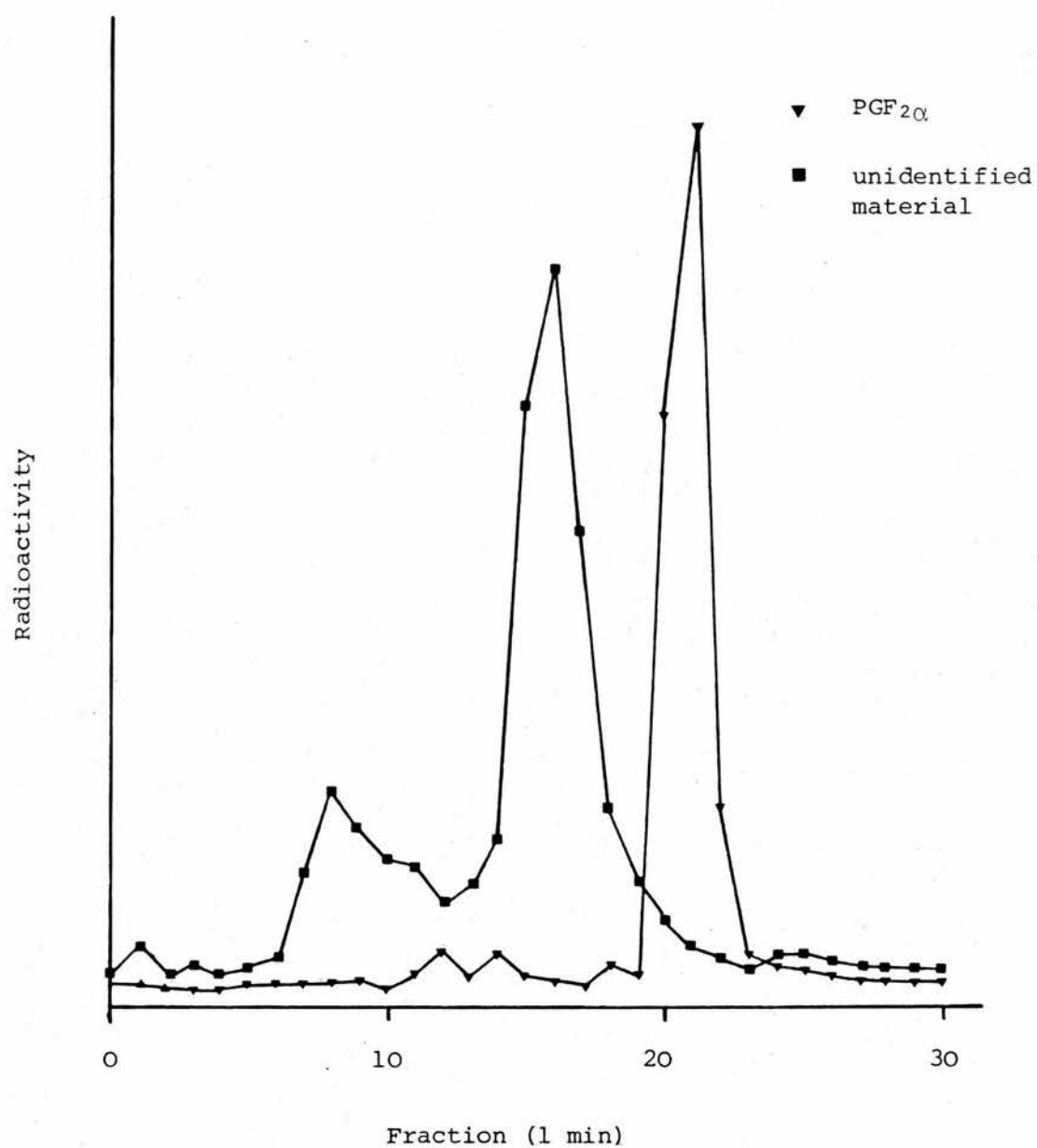


Figure 3.2 HPLC radio-chromatogram of unidentified material and PGF₂α both as their methyl ester derivatives

material. This was in contrast to the horse platelet incubates where the unidentified material was consistently present in considerably greater amounts than thromboxane B_2 .

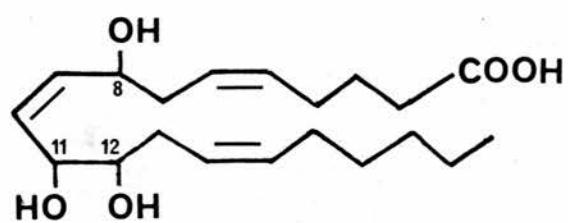
The formation of this unidentified material was not inhibited by the prostaglandin cyclo-oxygenase inhibitor indomethacin ($10\text{ }\mu\text{M}$), which inhibited the thromboxane B_2 formation, and indicated that the unknown was not a product of the prostaglandin cyclo-oxygenase pathway. However, its formation was inhibited by heating the platelets at 60°C for 20 min prior to incubation or by pre-incubation of the platelets with eicosa-5,8,11,14-tetraenoic acid (ETYA) ($10\text{ }\mu\text{M}$), an inhibitor of both prostaglandin cyclo-oxygenase and platelet and soyabean lipoxygenase enzymes (Downing, Barve, Gunstone, Jacobsberg and Lie Ken Jie, 1972; Hamberg and Samuelsson, 1974a).

Initially, purification of the methyl ester of the horse platelet incubation extracts was achieved by liquid-gel partition chromatography (solvent system LGC 1) and high performance liquid chromatography (solvent system HPLC 1). This afforded a single zone of radioactivity, slightly less polar than $\text{PGF}_{2\alpha}$ (Figure 3.2) containing the unidentified material of interest. A variety of derivatives were prepared and examined by GC-MS. The principal information derived from each is summarised in Table 3.1. Incubation of dihomo- γ -linolenic acid with washed human

TABLE 3.1

Summary of GC-MS results

Derivative	Carbon Value (3% OV-1 column)		Main information obtained from mass spectra
Methyl ester/trimethylsilyl ether (Me/TMS)	23.6	23.8	Indicated a compound with molecular weight of 584. The base peak at m/e 213 and the large ion at 243 indicated the most likely positions of the hydroxyl groups.
Methyl ester/butyl oxime/trimethylsilyl ether (Me/BuO/TMS)	23.6	23.8	Oximation had no effect on the material and indicated that there was no keto or hemiacetal moiety present.
Ethyl ester/trimethylsilyl ether (Et/TMS)	24.3	24.5	m/e 213 still the base peak. The ion fragment at m/e 243 was shifted to m/e 257 and indicated that the ion fragment at m/e 243 in the Me/TMS contained the ester group. Similarly the other ion fragments in the Me/TMS spectrum which contained the ester group were determined.
Methyl ester/hydrogenation/trimethylsilyl ether (Me/ H_2 /TMS)	24.4	24.7	Indicated that three double bonds were present in the original material.
Methyl ester/hydrogenation/n-butyl boronate/trimethylsilyl ether (Me/ H_2 /n-BuB/TMS)	26.0	26.2	All the material formed a boronate which indicated that a 1,2 or 1,3 diol was present in the material. Large ions were present at m/e 215 and 245.
Analogous material from di-homo- γ -linolenic acid incubate (Me/TMS)	23.9	24.1	Large ion fragments at m/e 213 and 245 indicated that the ion fragment at m/e 243 in the arachidonic acid incubate Me/TMS spectrum contained the 5,6 double bond whereas the ion fragment at m/e 213 did not.



and

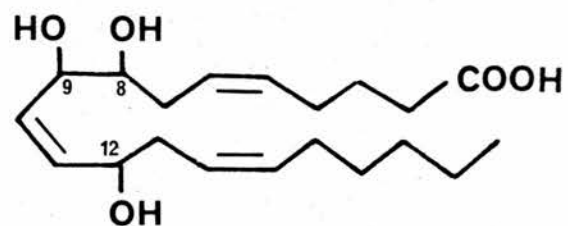


Figure 3.3 The most likely structures of the unidentified material

platelets resulted in the formation of an analogous material which provided additional structural information.

The unidentified material showed no diene absorption in the ultraviolet region ($100 \mu\text{g}.\text{ml}^{-1}$ in methanol) which indicated that no conjugated double bonds were present.

In all cases, the chromatographic peak shape of the derivatised material was not symmetrical (for example, peak 3 in Figure 3.1) and indicated that two partially resolved components were present. Mass spectra obtained from these partially resolved peaks at the carbon values indicated in Table 3.1 were very similar. Selected ion monitoring of the major ion fragments in the spectra demonstrated that they followed the same chromatographic profile as the total ion chromatogram and suggested that isomers of this material were present. It was noteworthy that since the partially resolved peaks were still present after hydrogenation they were not attributable to geometrical double bond isomers of the material.

All the evidence was consistent with a C20 fatty acid containing three double bonds and three hydroxyl groups. The Me/TMS, Et/TMS and Me/H₂/TMS derivatives indicated that the two most likely structures were with hydroxyl groups in the C-8, C-11 and C-12 or C-8, C-9 and C-12 positions and that both were probably present (Figure 3.3). Following examination of the spectra of the Me/H₂/n-BuB/TMS derivative, there was strong evidence that both these

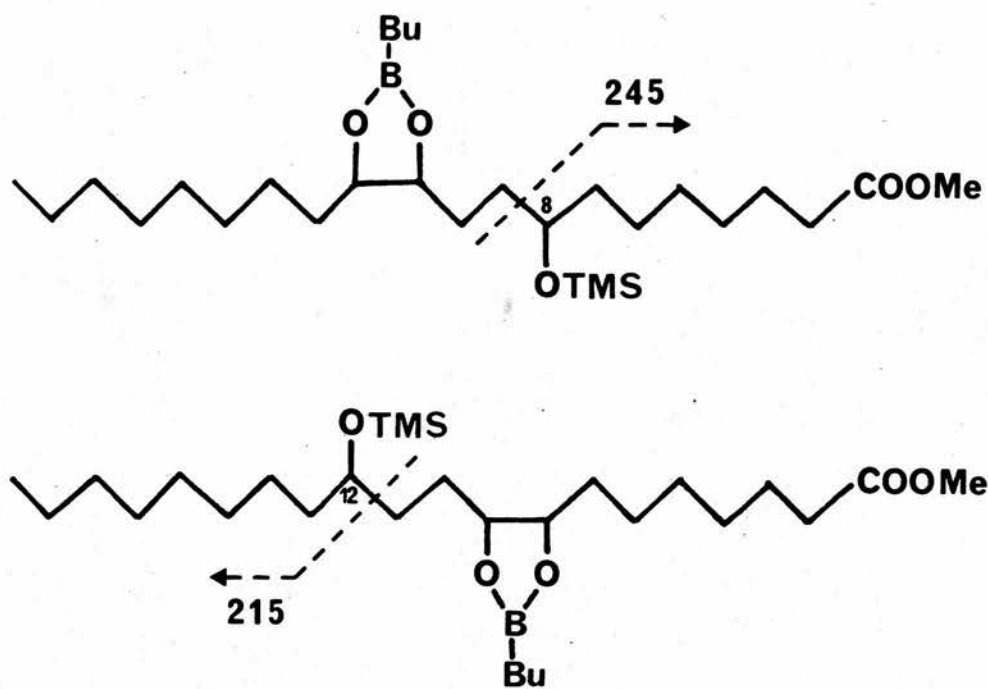


Figure 3.4 Proposed fragmentation of Me/n-BuB/TMS derivative

trihydroxy acids were present since large ions were present at m^+/e 215 and 245. These ion fragments were assigned as arising from the cleavage beside the only remaining TMS group in this derivative (Figure 3.4). Additional evidence for the presence of both trihydroxy acids was provided by the observation that the ratio of the ion fragments at m^+/e 213 and 243 in the Me/TMS spectra in different platelet incubates was not constant. It would be expected that cleavage would occur preferentially between the vicinal TMS groups (Capulla and Zorzut, 1968); thus the ion fragment at m^+/e 213 would arise predominantly from the 8,11,12-compound and the ion fragment at m^+/e 243 from the 8,9,12-compound (Figure 3.5). Therefore, if differing relative amounts of the two trihydroxy acids were formed in different incubations, the ratio of these two ion fragments would be expected to vary.

However, the presence of two positional isomers did not appear to account for the partially resolved gas chromatographic peaks. Selected ion monitoring of ions which were proposed to arise from different positional isomers, for example, the ion fragments at m^+/e 213 and 243 in the Me/TMS spectrum and the ion fragments at m^+/e 215 and 245 in the Me/H₂/n-BuB/TMS spectrum indicated that these ion fragments followed virtually identical chromatographic profiles. The partially resolved peaks were present in each case.

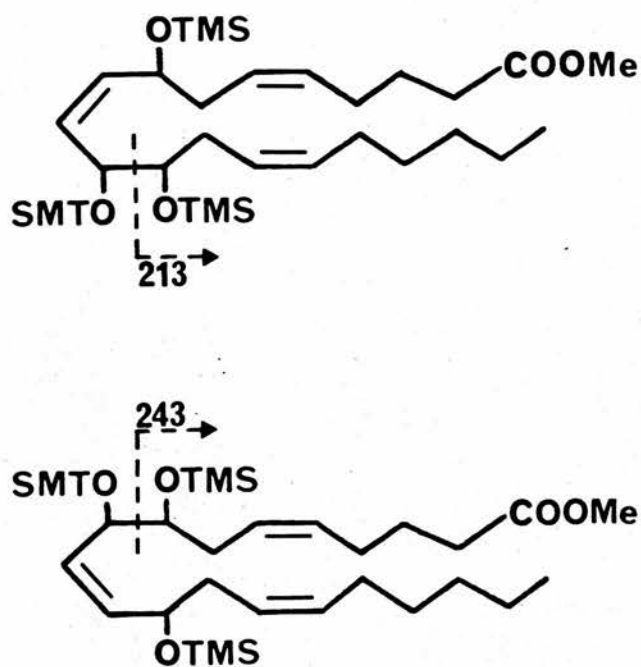


Figure 3.5 Proposed fragmentation of Me/TMS derivative

Evidence at this stage indicated that two positional trihydroxy trienoic acids (termed THETA) were formed and that hydroxyl stereoisomers of each were present. The following sub-section describes the separation of these positional isomers by liquid-gel partition chromatography. As a result, a simplified structure elucidation of the components became possible. Full mass spectral evidence is presented only for the separated components to avoid unnecessary repetition.

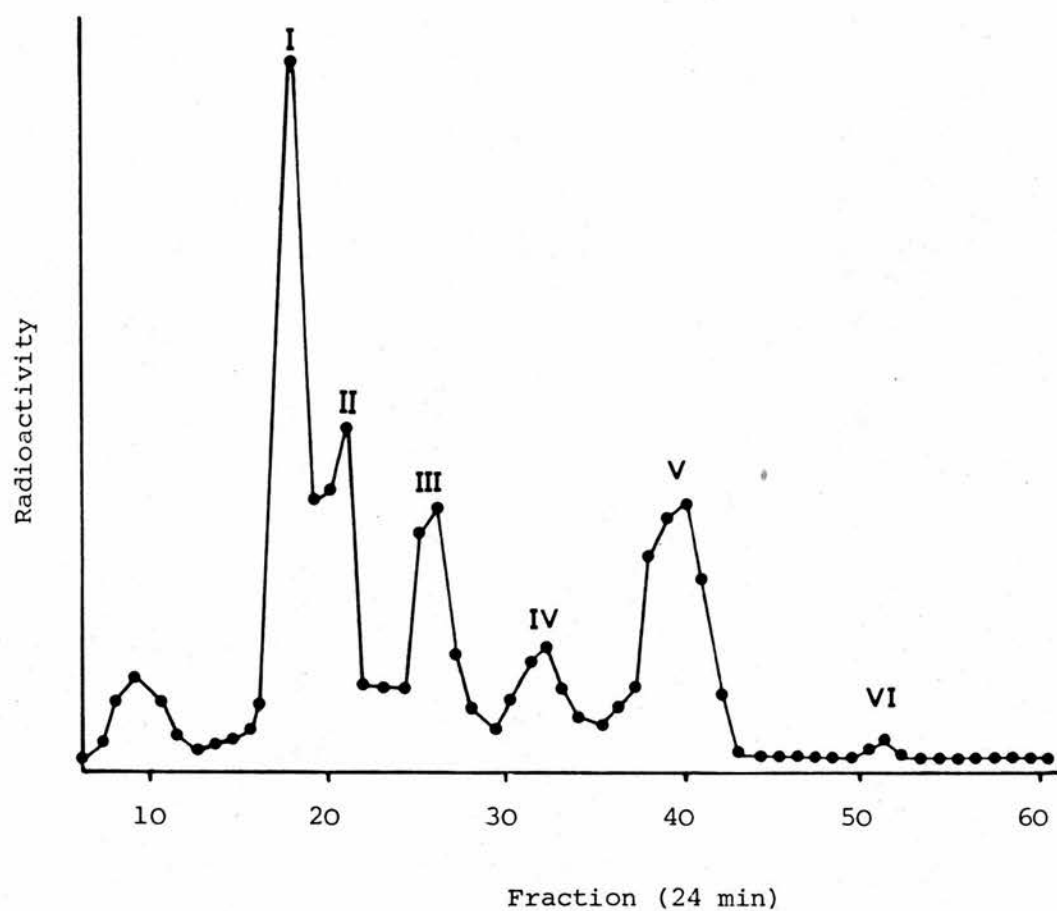


Figure 3.6 LGC radio-chromatogram of 33% aqueous ethanol extract from washed human platelet incubate sample

3.3 SEPARATION OF THE POSITIONAL ISOMERS
OF THE TRIHYDROXY ACIDS BY LIQUID-GEL
CHROMATOGRAPHY AND EXAMINATION OF A VARIETY OF
DERIVATIVES OF EACH BY GAS CHROMATOGRAPHY-
MASS SPECTROMETRY

Supplies of horse blood were not readily available and it was found that three day old human platelet concentrate obtained from the Blood Transfusion Service, Edinburgh Royal Infirmary (which was readily available) contained sufficient synthetic activity for the preparation of THETA. This concentrate was used for the preparation of THETA on several occasions as suitably large numbers of platelets were available, which allowed large scale incubations and hence the isolation of greater amounts of material than could be obtained using smaller volumes of fresh human blood donated by individual volunteers.

In order to attempt the separation of the two postulated positional isomers, the free acid from the incubation extract was subjected to liquid-gel partition chromatography (solvent system LGC 2). Figure 3.6 shows a typical chromatographic separation obtained from a human platelet incubate (using three day old platelet concentrate from the Blood Transfusion Service).

Examination of Zone V by GC-MS as the Me/TMS derivative indicated that three significant GC peaks were present

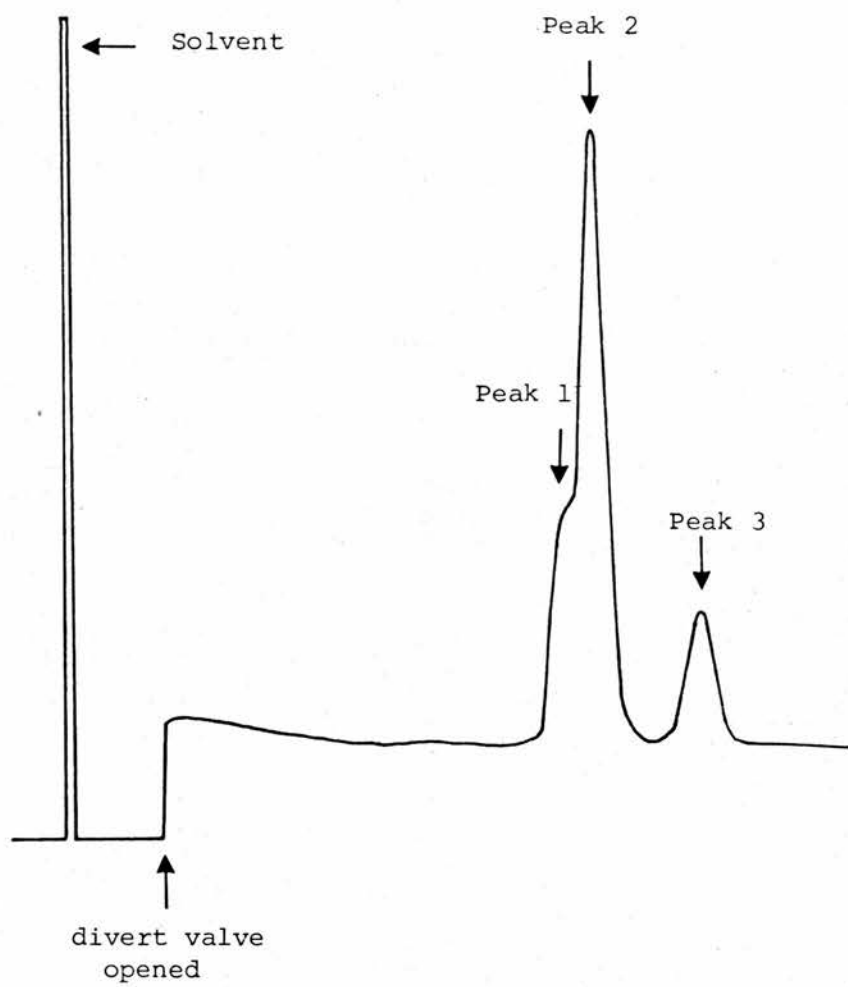
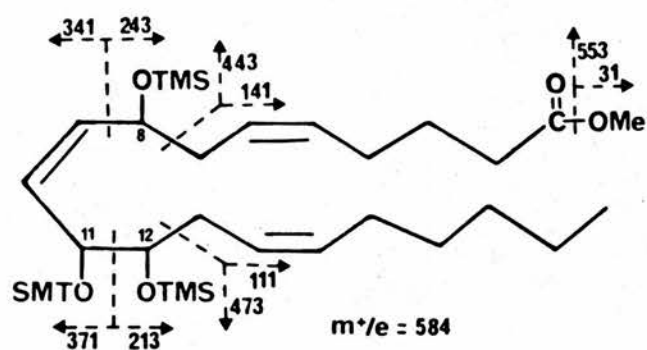
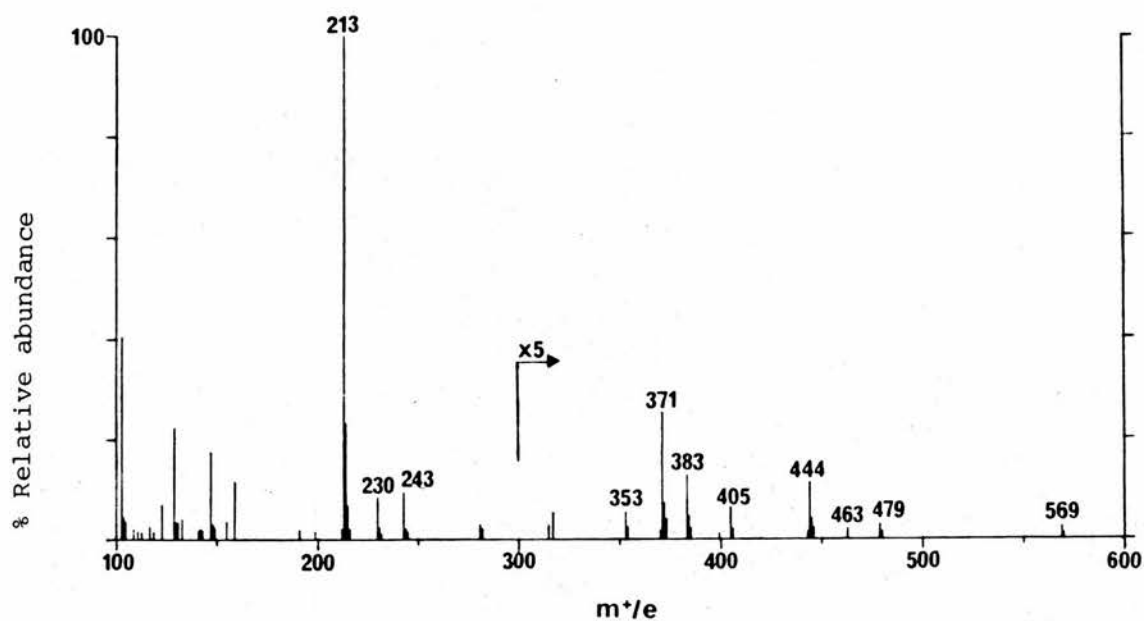


Figure 3.7 Gas chromatogram of LGC Zone V (Me/TMS)



(-TMSOH = 90)
(-CH₃ = 15)

Figure 3.8 Mass spectrum of 8,11,12-THETA (Me/TMS)

(Figure 3.7). The mass spectrum of peak 3 (carbon value 24.6) corresponded to thromboxane B₂. The mass spectrum was very similar to published data (ref. Hamberg, Svensson and Samuelsson, 1975; Smith, Harland and Brooks, 1977) and was later shown to be identical with a thromboxane B₂ standard donated in the course of this study by the Upjohn Company. The other major GC peaks (peaks 1 and 2) were only partially resolved (carbon values 23.6 and 23.8) and mass spectra taken at these carbon values were very similar (Figure 3.8). These spectra were noticeably different from spectra taken at the same carbon values in a sample of the material which had not been subjected to the chromatographic separation. The immediately apparent difference was the change in relative abundance of the ion fragments at m^+/e 213 and 243 as the ion fragment at m^+/e 243 was of much lower relative abundance in the LGC chromatographed sample. This indicated that separation of the proposed positional isomers had occurred. The spectra were consistent with isomers of a C₂₀ fatty acid methyl ester containing three double bonds and three TMS groups at the C-8, C-11 and C-12 positions. This compound was named 8,11,12-THETA standing for trihydroxy-eicosatrienoic acid.

Evidence for the isomers was provided by selected ion monitoring of the ion fragments at m^+/e 213 and 243 - their chromatographic profile was identical both to each other and to the total ion chromatogram and showed two

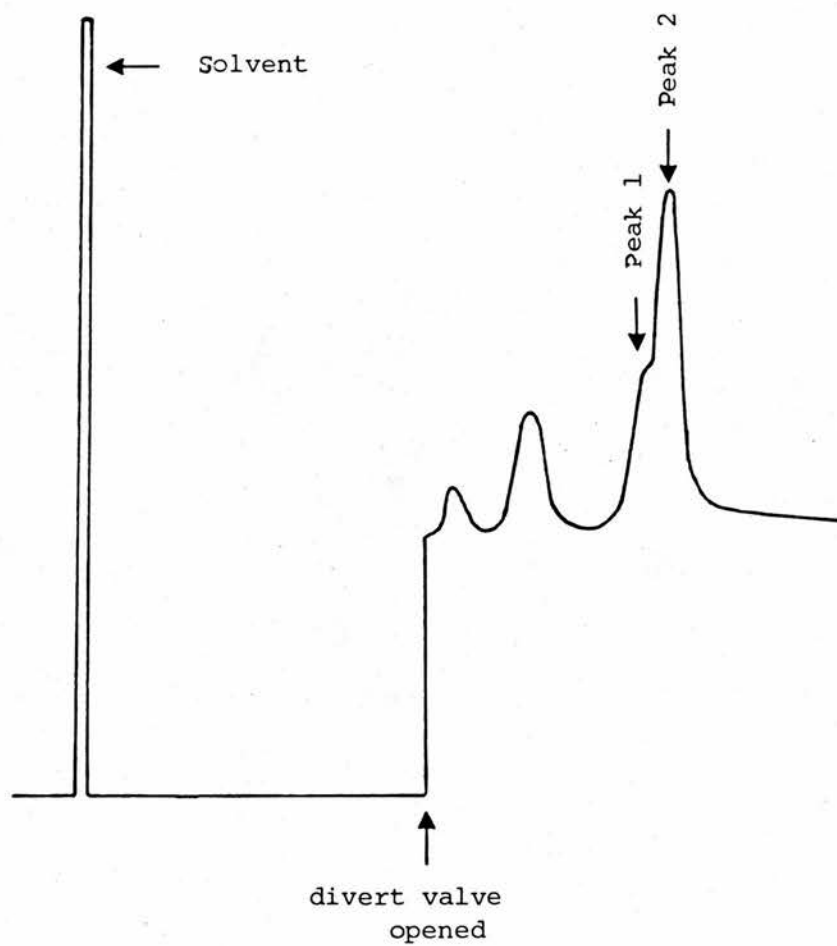


Figure 3.9 Gas chromatogram of LGC Zone IV (Me/TMS)

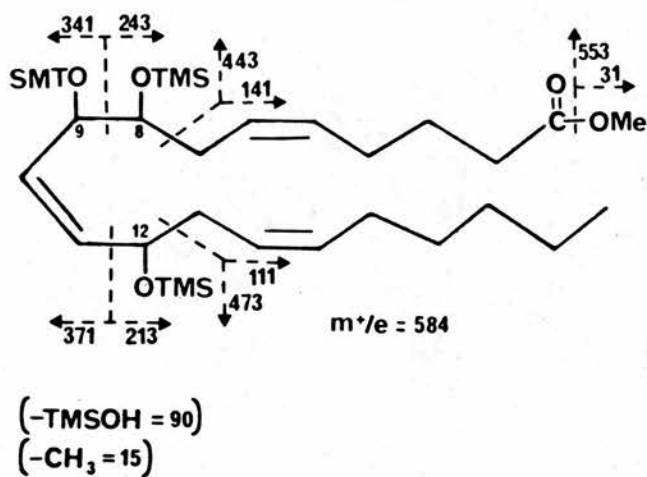
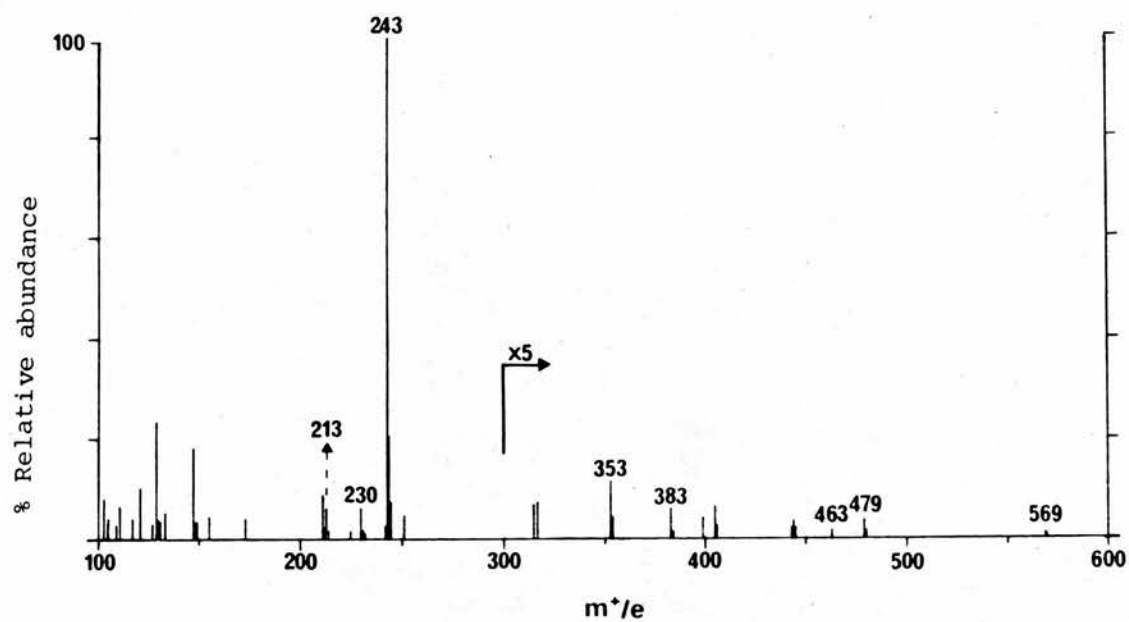


Figure 3.10 Mass spectrum of 8,9,12-THETA (Me/TMS)

partially resolved components. The base peak at m^+/e 213 was interpreted to arise from the preferential cleavage between the vicinal TMS groups (similar to that described by Capulla and Zorzut, 1968), whereas, the very much less intense ion fragment at m^+/e 243 was interpreted to arise from the less preferred cleavage at a TMS group allylic to a double bond. Most of the ion fragments were interpreted as resulting from cleavages at either side of the TMS groups or involving losses of or from TMS groups or loss of the methoxy moiety from the ester (the Et/TMS spectrum was consistent with all the assignments in Figure 3.8). An interesting exception was the fairly intense ion fragment at m^+/e 444 in the Me/TMS spectrum which was shifted to m^+/e 458 in the Et/TMS spectrum. A possible interpretation of these ions was that they resulted from a re-arrangement which involved the migration of the vicinal TMS group in the C-12 position and resulted in the loss of an unsaturated aldehyde ($\text{CH}_3-(\text{CH}_2)_4-\text{CH}=\text{CH}-\text{CH}_2-\text{CHO}$). This re-arrangement has been observed in the spectrum of 9,10-bis(TMS)-methyl stearate (Odham and Stenhagen, 1972).

GC-MS examination of Zone IV of the liquid-gel chromatographic separation as the Me/TMS derivative indicated that two partially resolved GC peaks (carbon values 23.6 and 23.8) were present (Figure 3.9). As above, the mass spectrometric analysis indicated that the partially resolved chromatographic peaks were isomers with very similar mass spectra (Figure 3.10). These were consistent

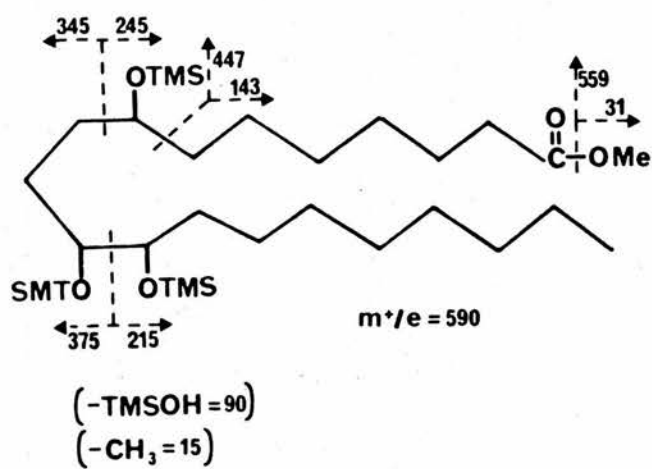
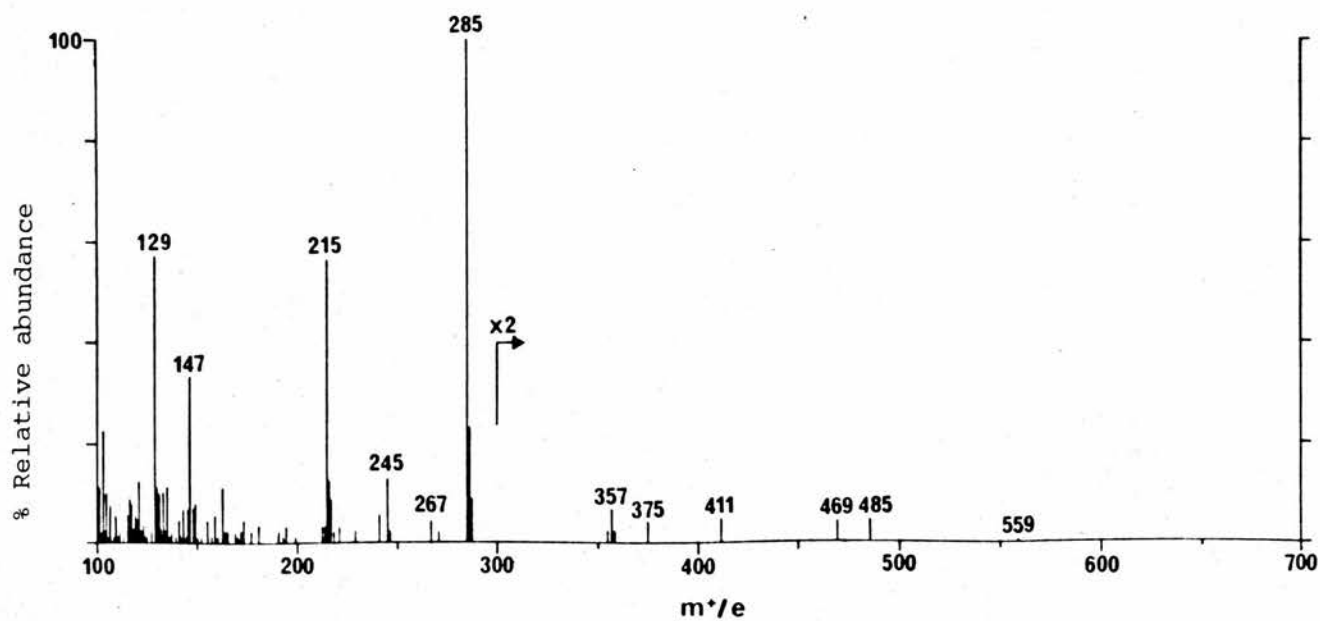


Figure 3.11 Mass spectrum of 8,11,12-THETA (Me/H₂/TMS)

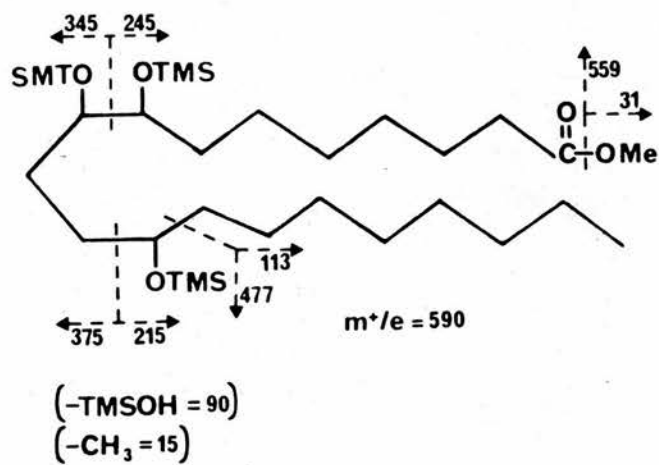
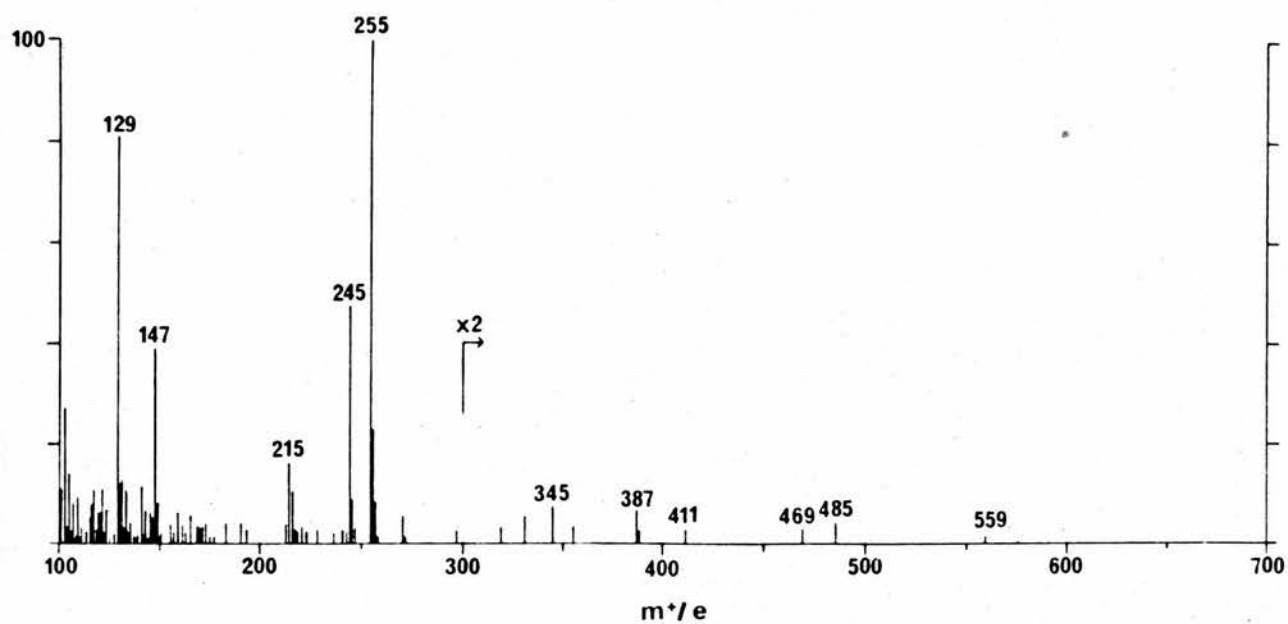


Figure 3.12 Mass spectrum of 8,9,12-THETA (Me/H₂/TMS)

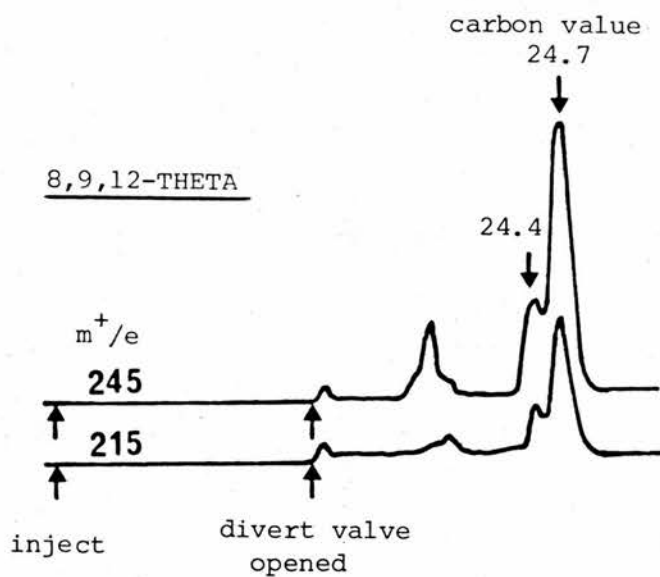
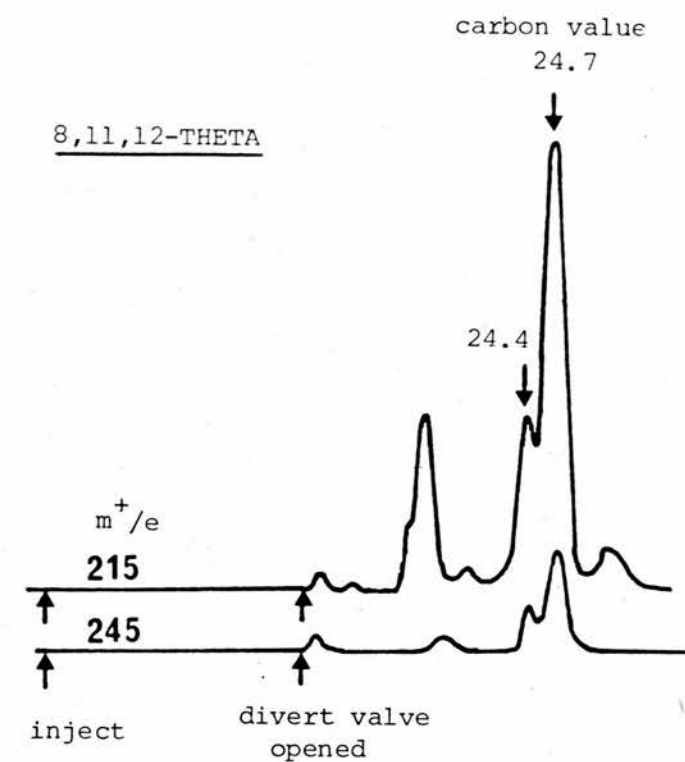


Figure 3.13 Selected ion chromatograms at m^+/e 215 and 245 of 8,11,12-THETA and 8,9,12-THETA (both Me/H₂/TMS)

with a C20 fatty acid methyl ester containing three double bonds and three TMS groups at the C-8, C-9 and C-12 positions. This compound was named 8,9,12-THETA. The base peak at m^+/e 243 was interpreted as arising from cleavage between the vicinal TMS groups (as in the case of 8,11,12-THETA) and it was noted that only a very small ion fragment at m^+/e 213 was present. The Et/TMS spectrum was consistent with all assignments.

The above evidence indicated that the two trihydroxy acids which had been postulated as products in sub-section 3.2 had been completely resolved from each other by the liquid-gel chromatographic separation. Further work was undertaken with the aim of confirming the structure of each of the trihydroxy acids by preparing a variety of derivatives for GC-MS analysis.

Following methylation, hydrogenation and TMS ether formation, both compounds still showed two partially resolved GC peaks (carbon values 24.4 and 24.7). The mass spectra of both compounds taken at these carbon values indicated that the partially resolved GC peaks were isomers and the spectra of both compounds confirmed the presence of three double bonds in the original materials. Mass spectra of the 8,11,12-THETA isomers and the 8,9,12-THETA isomers are shown in Figures 3.11 and 3.12 respectively. Selected ion monitoring of the ion fragments at m^+/e 245 and 215 of both compounds (Figure 3.13) supported the existence of isomers which were concluded to be hydroxyl diastereo-

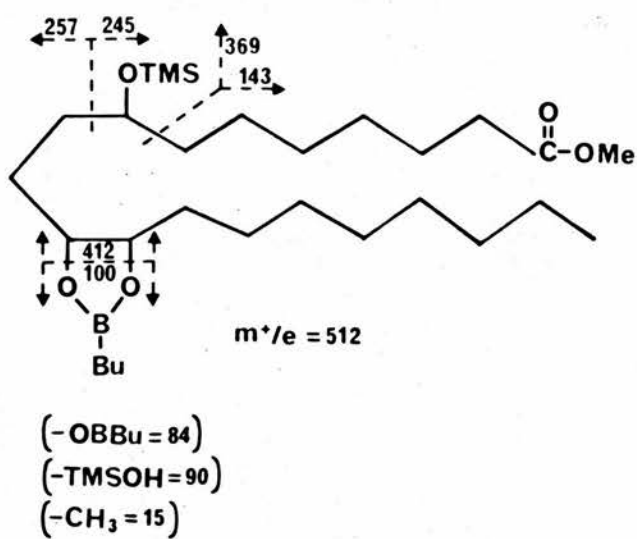
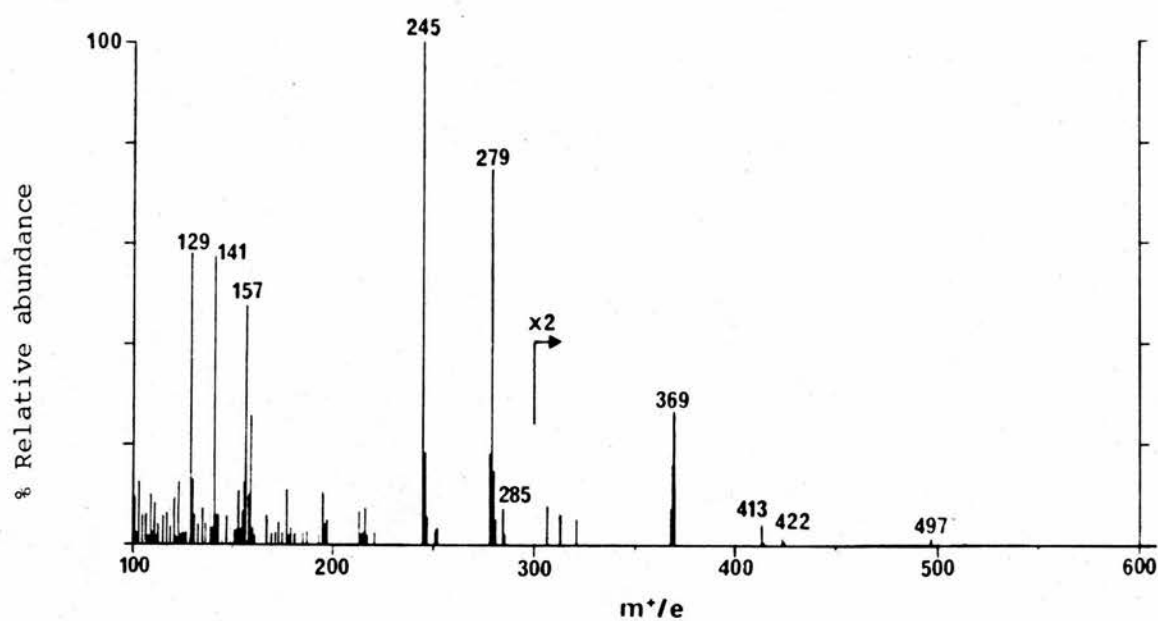


Figure 3.14 Mass spectrum of 8,11,12-THETA (Me/H₂/n-BuB/TMS)

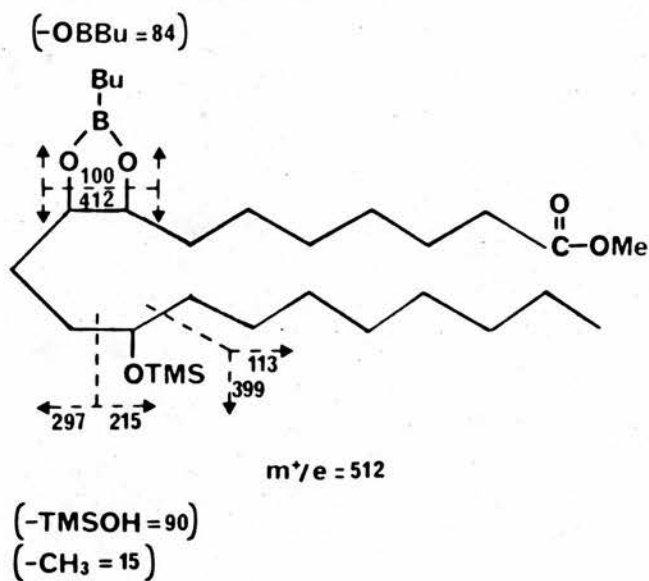
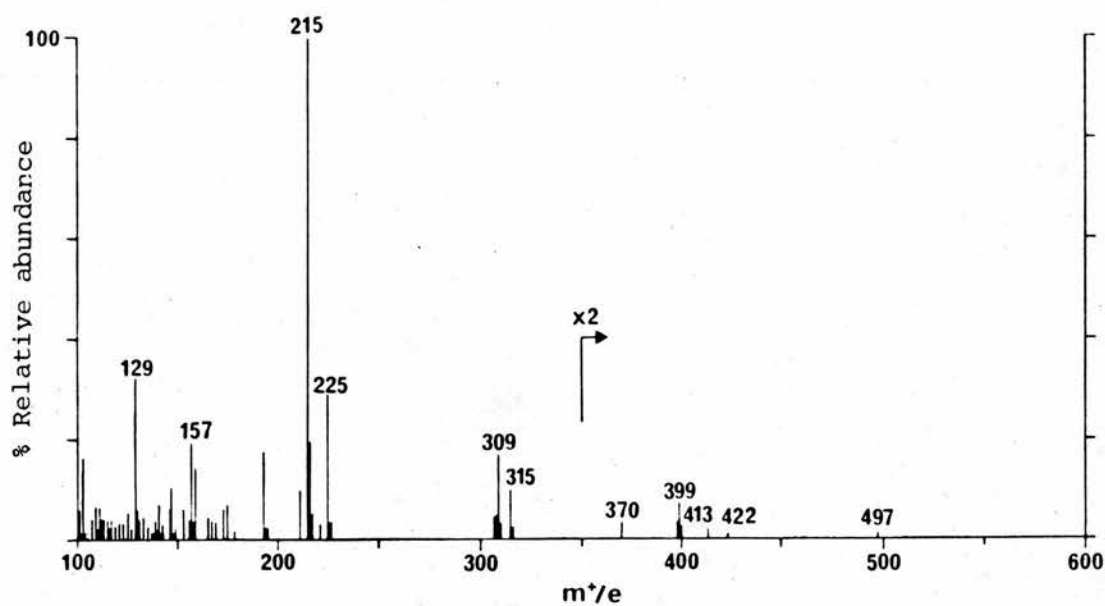


Figure 3.15 Mass spectrum of 8,9,12-THETA (Me/H₂/n-BuB/TMS)

isomers as removal of the double bonds had ruled out the possibility that the observed isomers were geometrical double bond isomers.

The hydrogenated forms of both compounds formed an n-butyl boronate/mono-TMS derivative which supported the presence of vicinal hydroxyl groups. Two partially resolved GC peaks were observed for both compounds (carbon values 26.0 and 26.2). Their mass spectra (Figures 3.14 and 3.15) indicated that the partially resolved chromatographic peaks were isomers which was supported by selected ion monitoring of the ion fragments at m^+/e 215 and 245. The ion fragment at m^+/e 245 was the base peak in the mass spectrum of the 8,11,12-THETA and the ion fragment at m^+/e 215 was the base peak in the mass spectrum of 8,9,12-THETA. These ion fragments were mutually exclusive for each positional isomer. Assignment of the ion fragments in these spectra was assisted by the boron isotope pattern (^{10}B 20%. ^{11}B 80%). The formation of the hydrogenated/boronated derivatives very effectively altered the position of preferential cleavage in these compounds from the vicinal hydroxyl groups (in the Me/TMS, Et/TMS, Me/H₂/TMS spectra) to the third remaining hydroxyl group and hence confirmed the position of this third hydroxyl group.

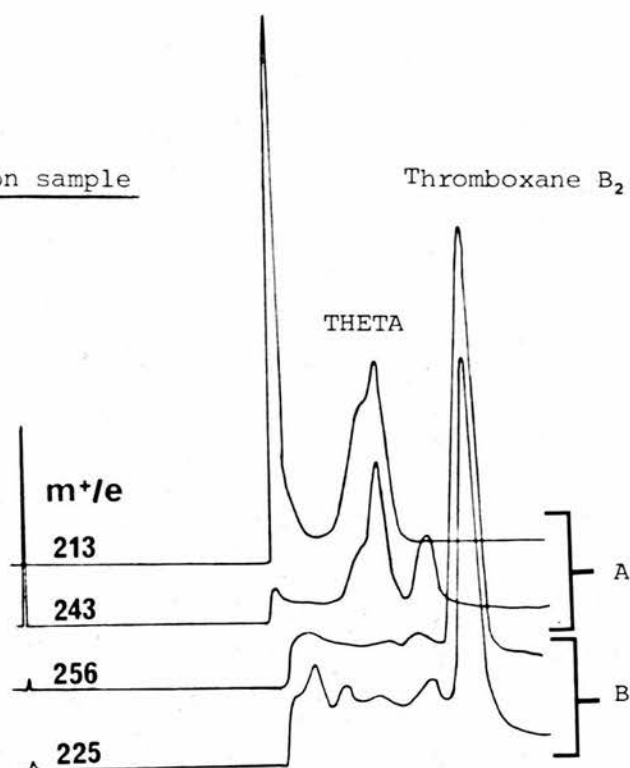
In summary, all GC-MS evidence supported the structure of two positional trihydroxy acids: 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid (8,11,12-THETA) and 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid (8,9,12-THETA). GC-MS

evidence indicated that hydroxyl stereoisomers of both these positional isomers were present.

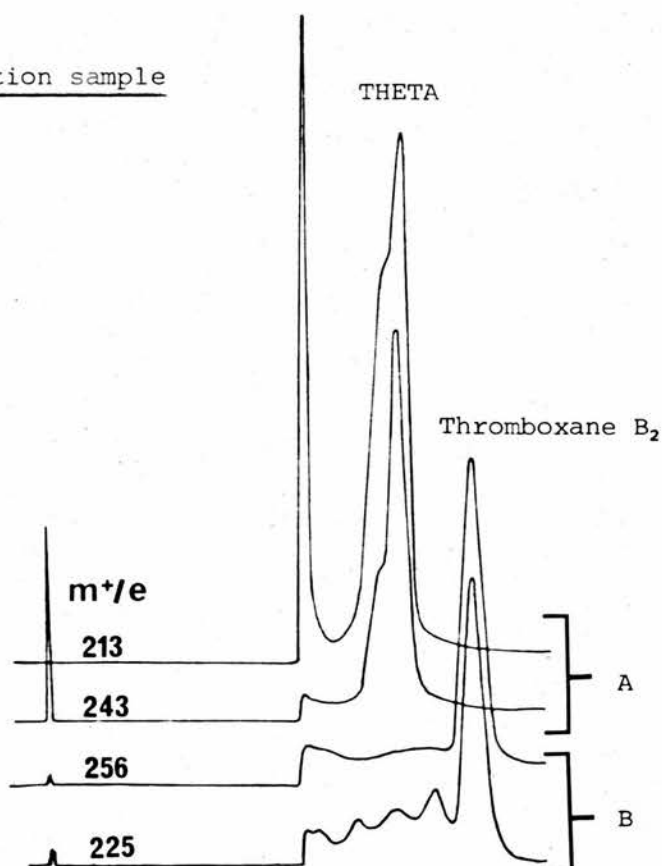
Further work is required to establish the configurations of the hydroxyl groups and double bonds. The reader is referred to the concluding remarks in this section for a consideration of the number of possible hydroxyl stereoisomers present and the configuration of the double bonds.

It is noteworthy that THETA solutions stored at -20°C in methanol were stable for several months.

5 minute incubation sample



30 minute incubation sample



A - monitor THETA

B - monitor thromboxane B₂

amplification constant
throughout

Figure 3.16 Selected ion chromatogram showing relative amounts of THETA and thromboxane B₂ in 5 min and 30 min incubation samples (Me/TMS)

It was demonstrated that in addition to washed platelets from horse and human, washed blood platelets from dog, cat and rabbit incubated with arachidonic acid produced THETA. Drs. Kerry, Poyser and Wilson detected THETA following incubation of arachidonic acid with sheep spleen and sheep uteri homogenates (Jones, Kerry, Poyser, Walker and Wilson, 1979). As reported earlier, the formation of these trihydroxy acids was not inhibited by the prostaglandin cyclo-oxygenase inhibitor indomethacin ($10\ \mu\text{M}$) but was almost completely abolished by the tetraynoic acid, ETYA ($10\ \mu\text{M}$) which is an inhibitor of both the prostaglandin cyclo-oxygenase and platelet lipoyxygenase enzymes.

It was interesting to observe that considerably more THETA was present after a 30 minute incubation compared with a 5 minute incubation period (Figure 3.16). A similar observation was made by Nugteren concerning 12-HETE synthesis by platelets. In contrast, it was observed that no additional thromboxane B_2 or HHT synthesis occurred after 5 minutes (Nugteren, 1977).

The substrate concentration of arachidonic acid was a crucial factor in the formation of the trihydroxy acids. At low concentrations of arachidonic acid, thromboxane B_2 was produced but the amounts of THETA were virtually

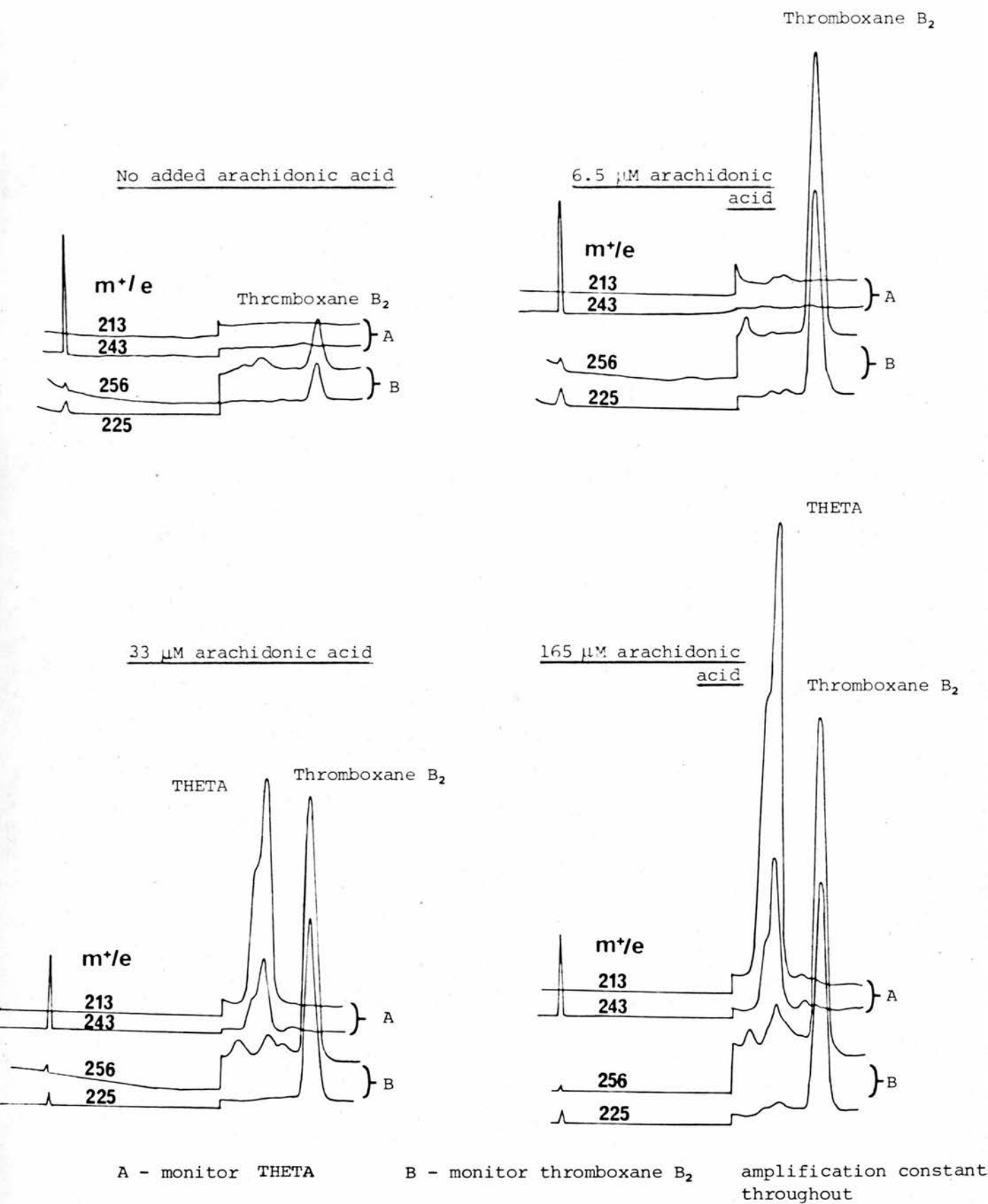


Figure 3.17 Selected ion chromatograms showing increased THETA production with increased substrate concentration in incubates (Me/TMS) from a washed human sample divided into four equal parts.

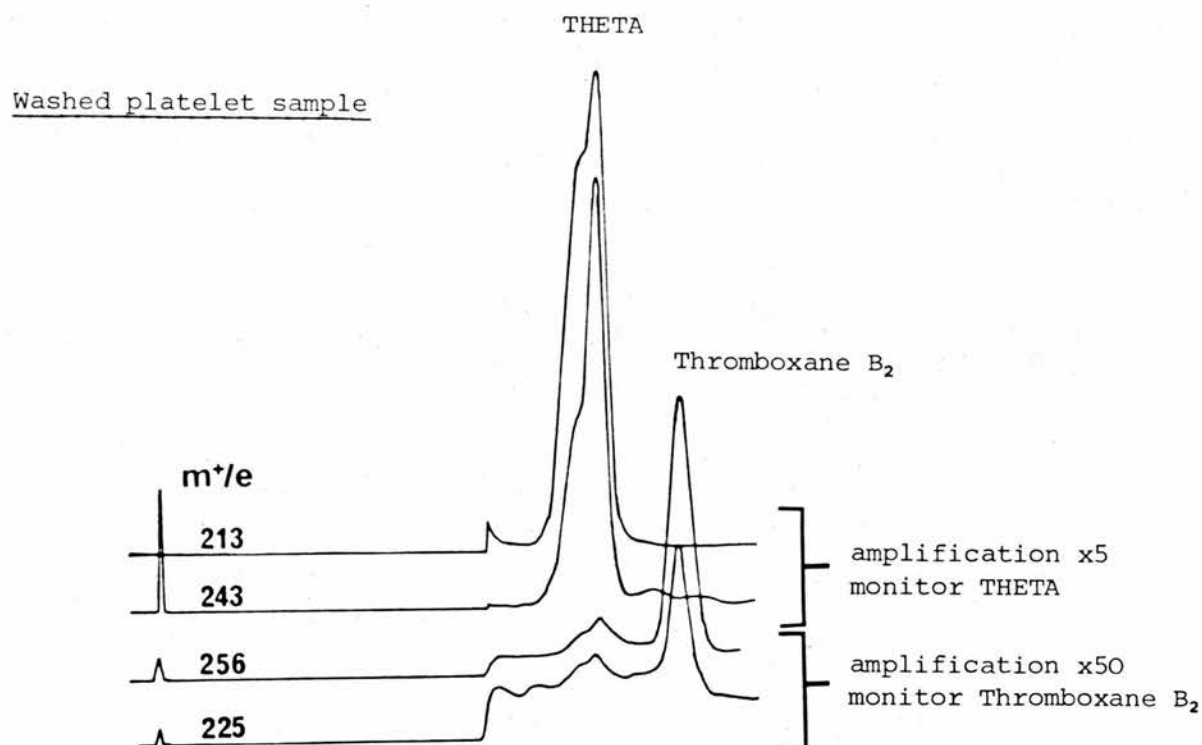
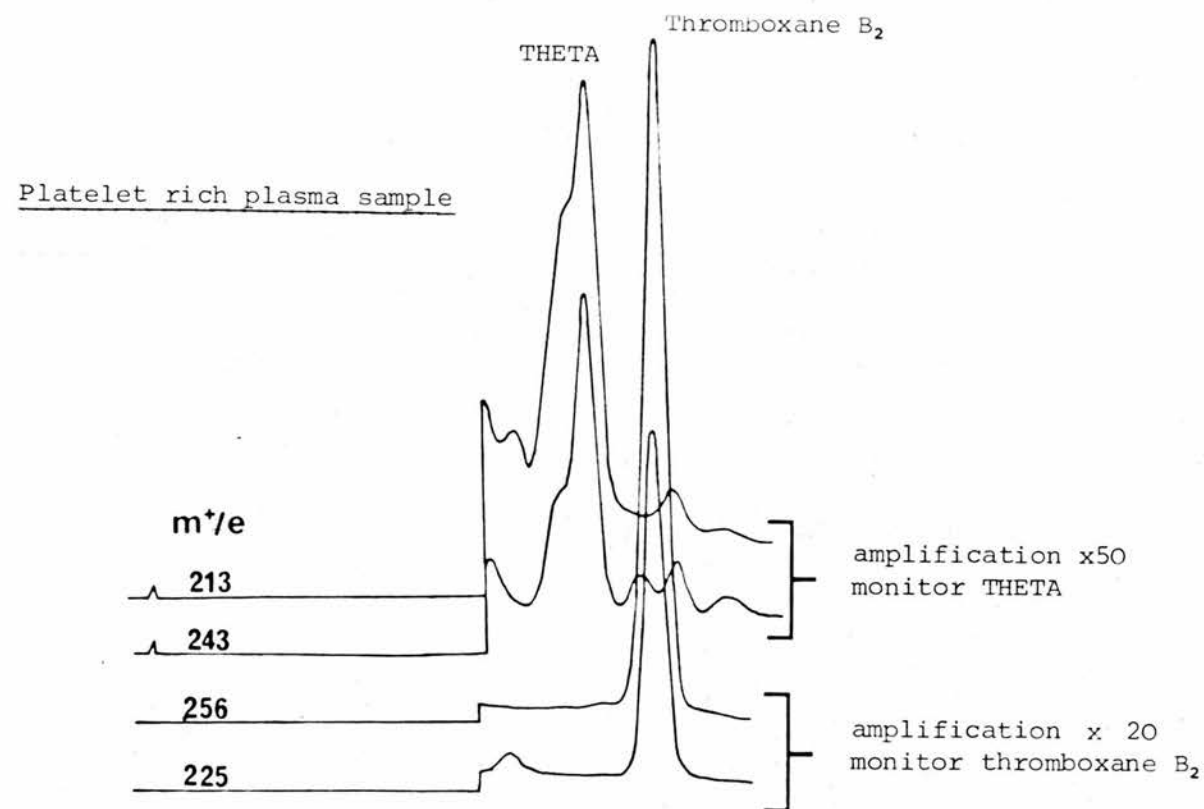


Figure 3.18 Selected ion chromatograms showing that THETA production was more pronounced in washed platelet than in platelet rich plasma incubate samples (Me/TMS). The reader is referred to Section 2.3 for full experimental details

non-detectable. As the concentration of arachidonic acid was increased, the relative amount of THETA compared to thromboxane B₂ increased dramatically (Figure 3.17).

The formation of THETA was more pronounced in washed platelets than in platelet rich plasma incubates (Figure 3.18). This was probably accounted for by the lower amounts of arachidonic acid available to the platelets in the platelet rich plasma as considerable protein binding of arachidonic acid occurs (Nugteren, 1977; Smith, Ingberman and Silver, 1977). This observation may therefore be related to the previous observation that THETA synthesis was more significant at higher arachidonic acid concentrations.

Shortly after the isolation and structural determination of THETA was reported from our laboratory (Jones, Kerry, Poyser, Walker and Wilson, 1978), Bryant and Bailey reported the isolation of the same compounds from washed platelet incubations (Bryant and Bailey, 1979). These workers deduced that 8,11,12-THETA was definitely present in their samples and that 8,9,12-THETA might also be present. No attempt was made to separate the positional isomers. They reported that addition of glucose (2 mg. ml⁻¹) to the platelet incubation buffer totally eliminated THETA production. This effect was explained by the glucose enhancing the level of reduced glutathione in the platelets which caused a lowering of the level of 12-HPETE by promoting its conversion into 12-HETE. Attempts to

confirm these results in this study, at glucose concentrations of 2 and 10 mg.ml⁻¹, did not result in the reported inhibition. The exact experimental detail was not provided in their publication and so differences in procedure may account for this conflicting result.

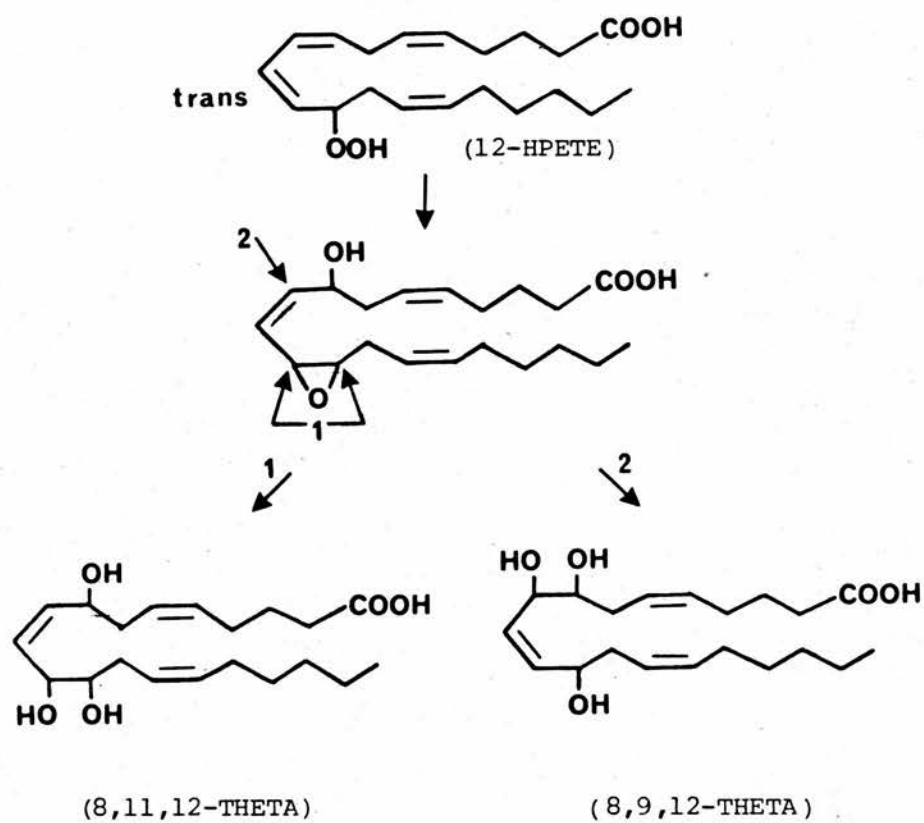


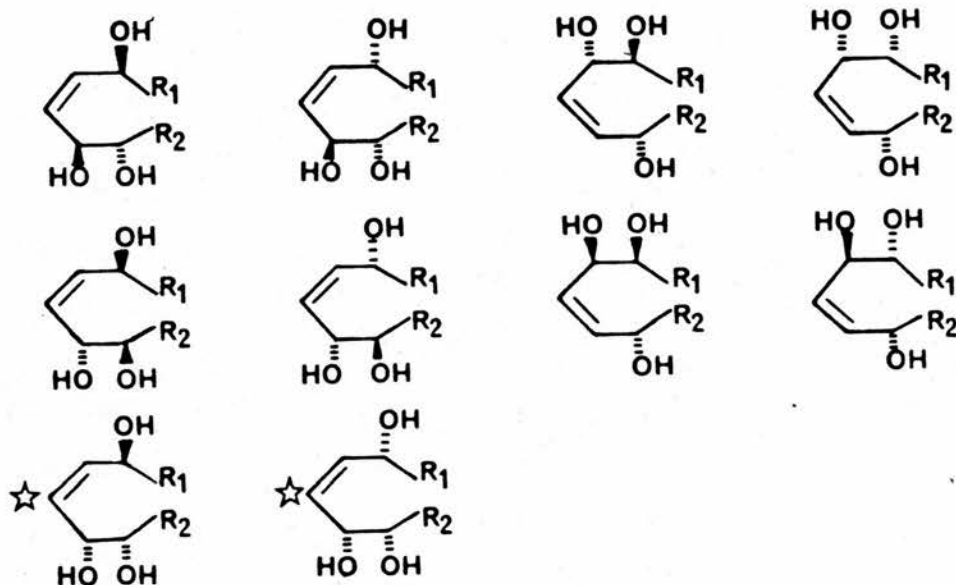
Figure 3.19 Proposed mechanism of THETA formation

Consideration was given to the mechanism of THETA formation. The formation of THETA did not occur by the prostaglandin cyclo-oxygenase pathway and it seemed likely that the trihydroxy acids were products of the platelet lipoxygenase pathway as ETYA inhibited both 12-HETE and THETA production. A possible mechanism involved further transformation of 12-HPETE via an epoxy-hydroxy intermediate (Figure 3.19). Hydrolytic ring opening of such an epoxide intermediate at the positions indicated in the figure would result in the formation of the two hydroxy acids. It was of interest to note that 9-hydroxy-12,13-epoxy-10-octadecadenoic acid has been reported to be converted into a mixture of 9,10,13-trihydroxy-11-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid in aqueous ethanol (Hamberg, personal communication). After consideration of the results of the experiment with differing arachidonic acid substrate concentrations, it seems likely that THETA formation occurs during high rates of 12-HPETE synthesis.

Several epoxy-hydroxy acids have been proposed and determined as intermediates resulting from transformations involving hydroperoxides in the synthesis of trihydroxy fatty acids in plant lipoxygenase systems (Graveland, 1970, 1973; Arens and Grosch, 1974; for reviews: Gardener, 1975; Veldink, Vliegenthart and Boldingh, 1977). In incu-

8,11,12-THETA

8,9,12-THETA



☆ = considered unlikely

Figure 3.20 Possible hydroxyl stereoisomers of 8,11,12-THETA and 8,9,12-THETA

bations of linoleic acid with flour water suspensions and doughs, Graveland (1970) isolated trihydroxy acids in both cases, but only in the latter case isolated the intermediate epoxy-hydroxy compound. He stated that, in the suspension, hydrolysis of the intermediate took place so rapidly it was impossible to detect. Trihydroxy acids have also been detected as products in non-enzymic reactions, for example, following treatment of linoleic acid hydroperoxide with ferric ions and cysteine (Gardner, Kleiman and Weisleder, 1974).

Assuming the proposed mechanism of THETA formation then several stereoisomers of both 8,11,12-THETA and 8,9,12-THETA were expected to be present (Figure 3.20). From a consideration of the literature, it was considered likely that the 9,10-double bond in 8,11,12-THETA and the 10,11-double bond in 8,9,12-THETA were trans, although from the evidence obtained, it was not possible to determine whether the configurations were trans or cis and the possibility that both were present could not be discounted. The 5,6- and 14,15-double bonds were considered likely to have remained cis.

In order to possibly detect and identify the epoxy-hydroxy or a similar intermediate, it was decided to investigate the less polar peaks obtained from the liquid-gel chromatographic separation. This work is described in section 4 of this thesis. It was also of interest to

determine whether or not 8,11,12-THETA and 8,9,12-THETA possessed any pharmacological activity and a number of tests were performed which are described in Section 5.

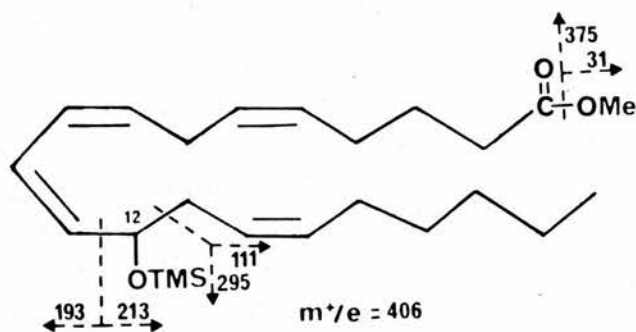
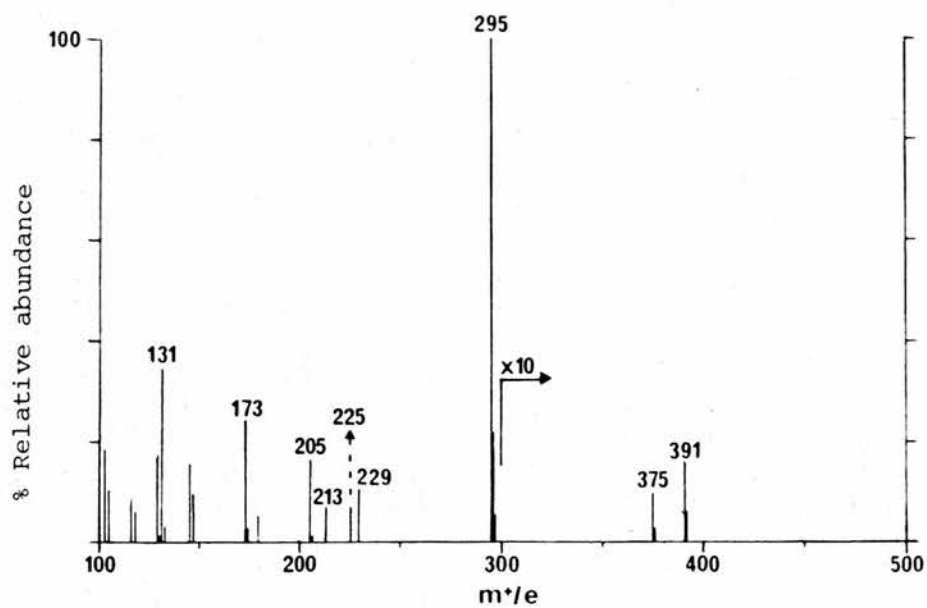
SECTION 4

THE INCUBATION OF ARACHIDONIC ACID
WITH WASHED BLOOD PLATELETS WITH THE
DETECTION AND SUBSEQUENT STRUCTURE ELUCIDATION
OF NOVEL METABOLITES

PART 2

During attempts to identify the proposed THETA intermediate (8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid) an analogous compound was detected. Structural examination of various derivatives of this compound by GC-MS indicated its structure as 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid and not the proposed THETA intermediate. Isomers of this compound were shown to be present and were resolved by high performance liquid chromatography.

Other products of the platelet incubation extract were identified by GC-MS and included the monohydroxytetraenoic acid 12-HETE, the C17 monohydroxy acid HHT and another unidentified compound which co-chromatographed on the liquid-gel column with HHT.



(-TMSOH = 90)
(-CH₃ = 15)

Figure 4.1 Mass spectrum of 12-HETE (Me/TMS)

4.2 THE EXAMINATION OF THE TWO LEAST POLAR
LIQUID-GEL CHROMATOGRAPHIC ZONES AND THE
DETECTION AND EXAMINATION BY GAS CHROMATOGRAPHY-
MASS SPECTROMETRY OF A MOLECULE ANALOGOUS TO
THE PROPOSED THETA INTERMEDIATE

Zone I of the Liquid-Gel Chromatographic Separation

The monohydroxytetraenoic acid 12-HETE was identified in Zone I of the liquid-gel chromatographic separation of the platelet incubate (Figure 3.6). The stability of the Me/TMS derivative of this material to gas chromatography was found to depend markedly on the chromatographic conditions. On many occasions, a broad irregularly shaped GC peak with higher carbon value (22.0 - 23.1) than that previously reported for 12-HETE (Me/TMS) (carbon value 21.3) was detected and was attributed to the "on-column" decomposition of the 12-HETE derivative. Decomposition was demonstrated by making repeat injections of the same sample under varying chromatographic conditions (injector/oven temperatures and column length/condition). Mass spectra of the higher carbon value GC peak contained a characteristic intense ion fragment at m^+/e 213. Although on many occasions the chromatography of the Me/TMS derivative was not totally satisfactory, it was considered adequate for qualitative identification. Under optimised chromatographic conditions, satisfactory chromatography of this derivative was achieved. Peak 1 (Figure 3.1) in the chromatogram of the Me/TMS derivative from a horse plate-

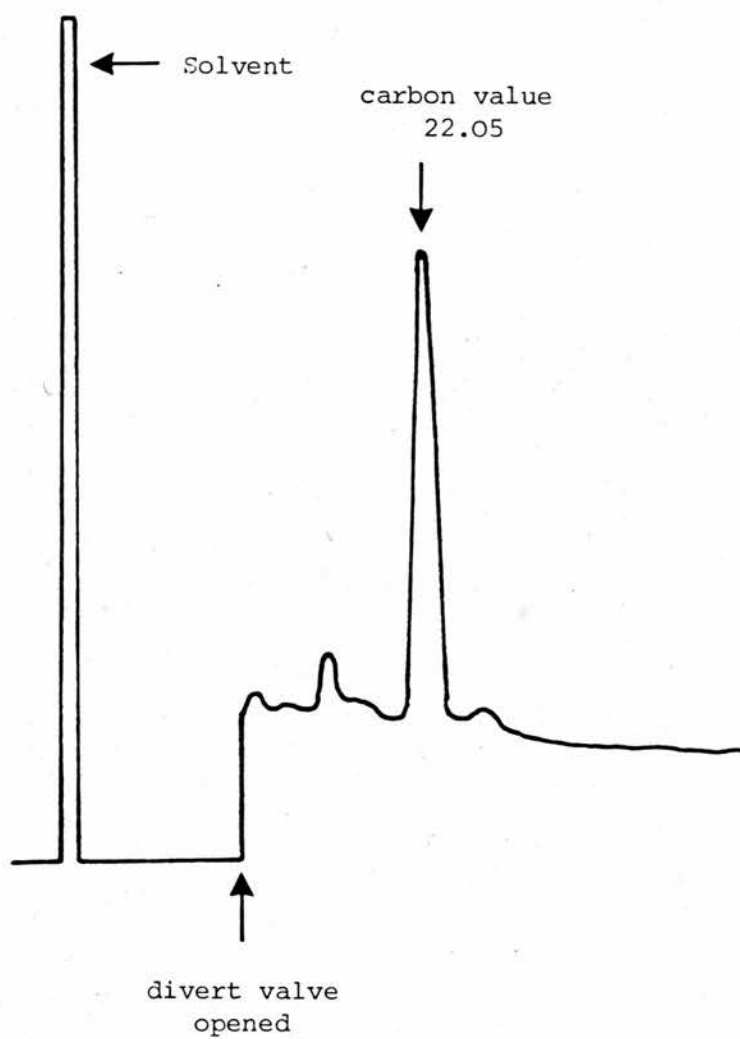


Figure 4.2 Gas chromatogram of LGC Zone II (Me/TMS)

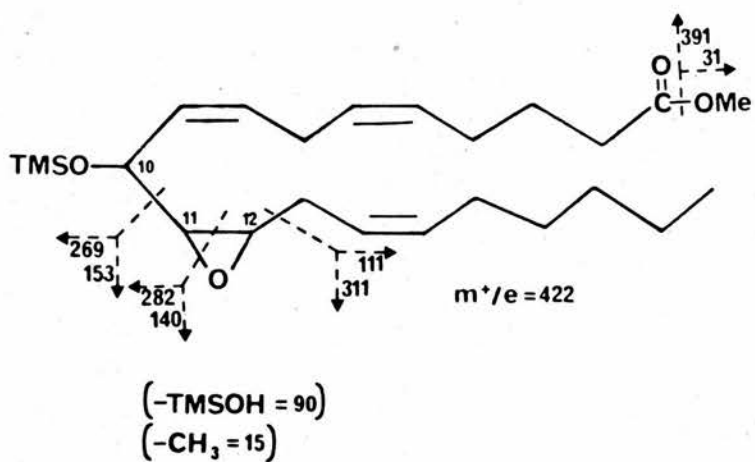
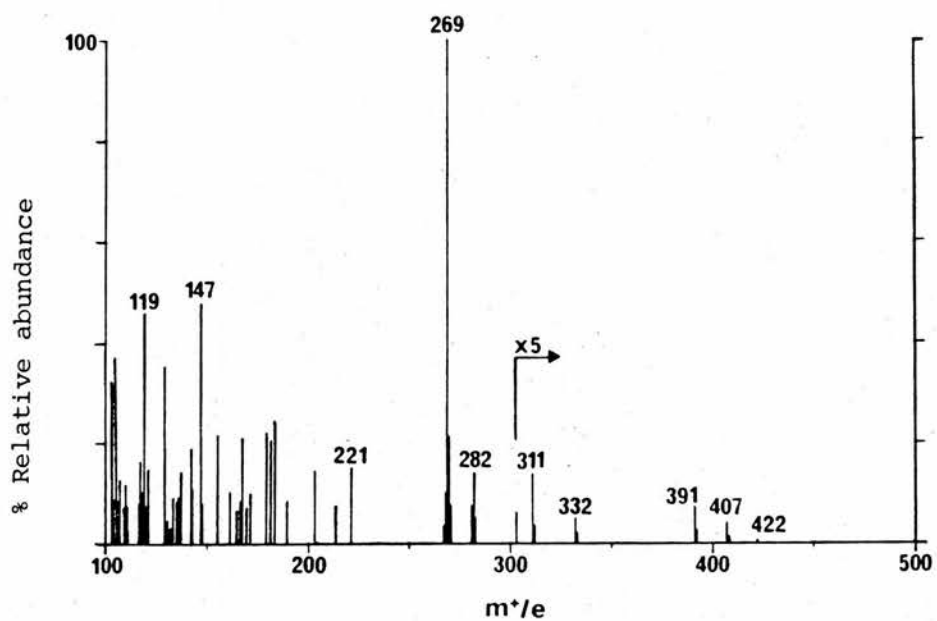


Figure 4.3 Mass spectrum of major peak from LGC Zone II
(Me/TMS)

let incubate had an identical carbon value (21.3) as that reported for 12-HETE (Me/TMS) and its mass spectrum (Figure 4.1) was very similar to published data (Hamberg and Samuelsson, 1974a).

Solutions of 12-HETE stored at -20°C in methanol were not stable. 12-HETE has a UV absorbing chromophore (attributable to the diene) which shows an absorbance maximum at 235 nm in methanol. Significant loss of this chromophore occurred after two to three weeks storage. Similar storage problems of 12-HETE have been reported by Turner and the Upjohn Company (Turner and Lynn, 1978).

Zone II of the Liquid-Gel Chromatographic Separation

Examination by GC-MS of the more polar shoulder on the 12-HETE peak of the liquid-gel chromatographic separation (Zone II, Figure 3.6) as the Me/TMS derivative indicated a single major GC peak (carbon value 22.05) (Figure 4.2) with a mass spectrum analogous to the proposed THETA intermediate. The mass spectrum (Figure 4.3) indicated a compound with a molecular weight of m^{+}/e 422 and was consistent with a C20 fatty acid methyl ester containing three double bonds, one TMS group and one keto or epoxy oxygen. The material did not form an oxime which indicated the absence of a keto group. The mass spectrum of the Et/TMS derivative (carbon value 22.6) was consistent with all the assignments in Figure 4.3 and contained a base peak at m^{+}/e

283. The Me/TMS and Et/TMS spectra indicated that the most likely position of the TMS group was in the C-10 position but did not rule out the possibility of a TMS group in the C-8 position. It was interesting to note that a C18 analogue with a TMS group adjacent to an epoxide showed a very similar fragmentation pattern (Hamberg and Gotthammar, 1973). In contrast, mass spectra of C18 γ -hydroxy epoxides, which were comparable to the proposed THETA intermediate, showed marked differences (Graveland, 1970; Hamberg, 1975). Notably cleavage was observed adjacent to the TMS group. The corresponding cleavage in the proposed THETA intermediate would result in an ion fragment in the Me/TMS spectrum at m^+/e 243 - this ion was totally absent from the spectrum of the platelet product. The ion fragments at m^+/e 282/281 in the Me/TMS spectrum of the platelet product were interpreted as resulting from the transannular cleavage of the epoxide which occurs with and without proton transfer (Budzikiewicz, Djerassi and Williams, 1967; Alpin and Coles, 1967).

This compound was named EPHETA standing for epoxyhydroxy-eicosatrienoic acid. Its formation was not inhibited by indomethacin (10 μ M) but was inhibited by ETYA (10 μ M). Further work was undertaken with the aim of establishing the position of the hydroxyl group, confirming the number of double bonds in the molecule and demonstrating whether or not an epoxide group was present.

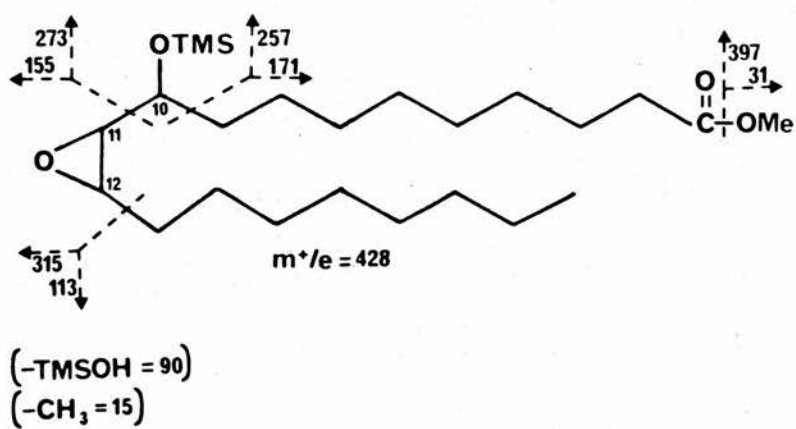
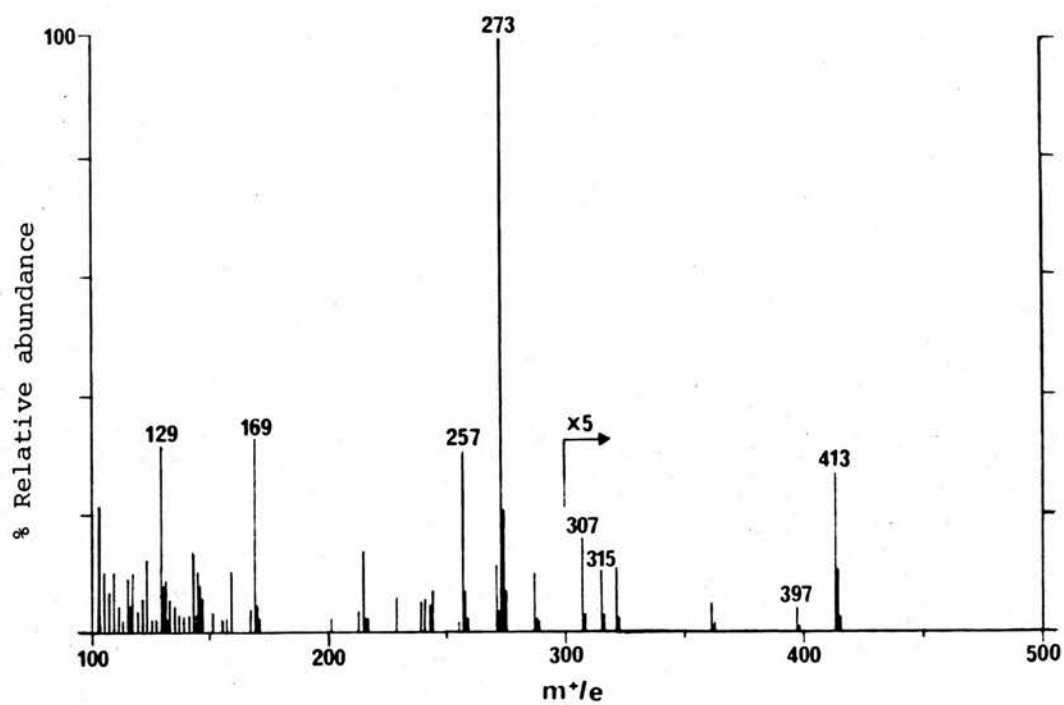


Figure 4.4 Mass spectrum of EPHETA (Me/H₂/TMS)

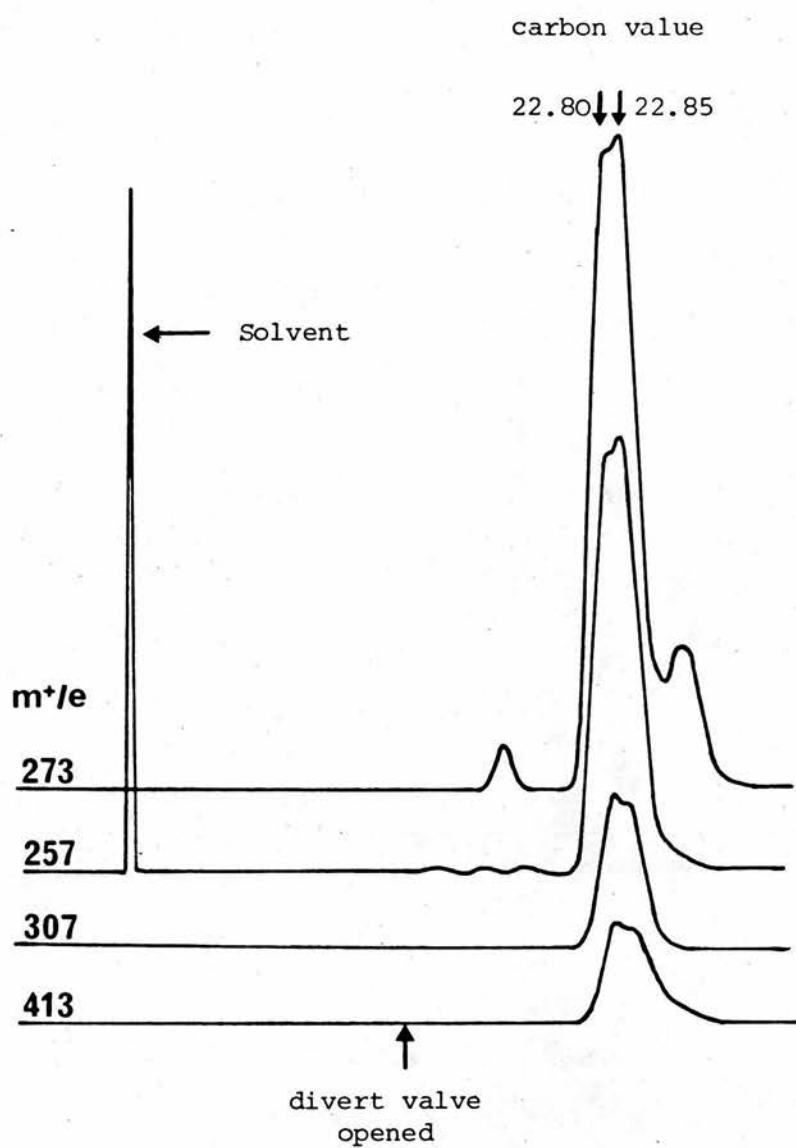


Figure 4.5 Selected ion chromatograms of EPHETA
(Me/H₂/TMS)

Following methylation, hydrogenation and TMS ether formation, the material showed two marginally resolved GC peaks (carbon values 22.80, 22.85). Mass spectra (Figure 4.4) taken at these carbon values were very similar and indicated the presence of three double bonds in the original material (the methyl ester of oleic acid epoxide was unaffected by the hydrogenation procedure so it was assumed that if an epoxide group was present in the molecule, it would be unaffected). In these mass spectra, the large ion fragments at m^+/e 273 and 257 provided strong evidence that the TMS group was in the C-10 position as these ions corresponded to cleavage at either side of a TMS group in this position. The mass spectra indicated that very small amounts of partially hydrogenated material may be present but this did not account for the two marginally resolved GC peaks, as the same GC profile as the total ion chromatographic profile was observed in the single ion chromatograms of the ion fragments at m^+/e 413, 307, 273 and 257 (Figure 4.5). This suggested that isomers of the molecule were present.

It is well known that lithium aluminium hydride (LiAlH_4) reduces an epoxide group to a mono-alcohol. Two positional isomers are usually formed (Hamberg and Gotthammar, 1973). During this reaction, an ester moiety would also be reduced to a primary alcohol. Gas chromatography of the TMS derivative of the product of the LiAlH_4 reaction showed a single peak (carbon value 23.0) whose mass spectrum was

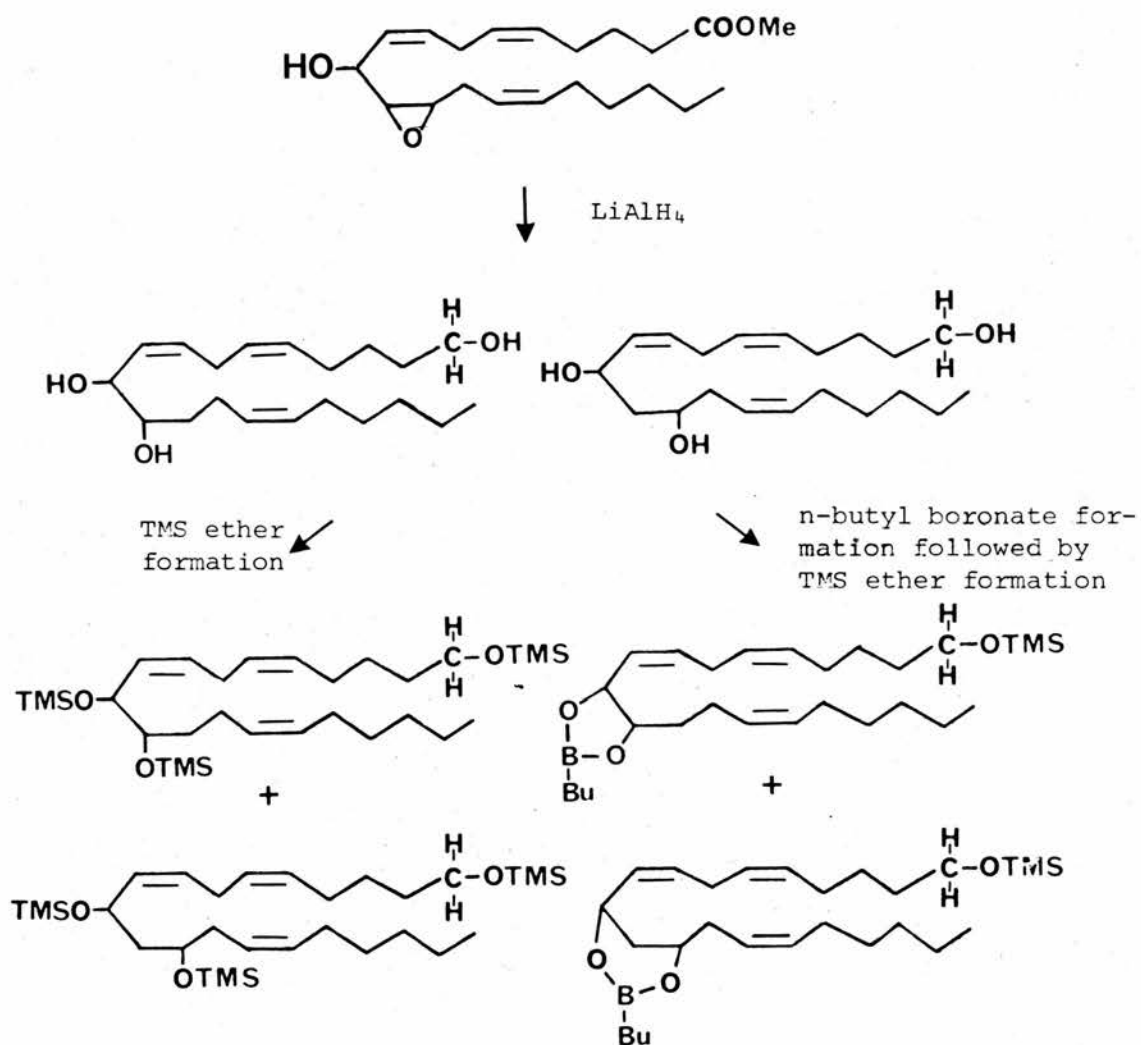
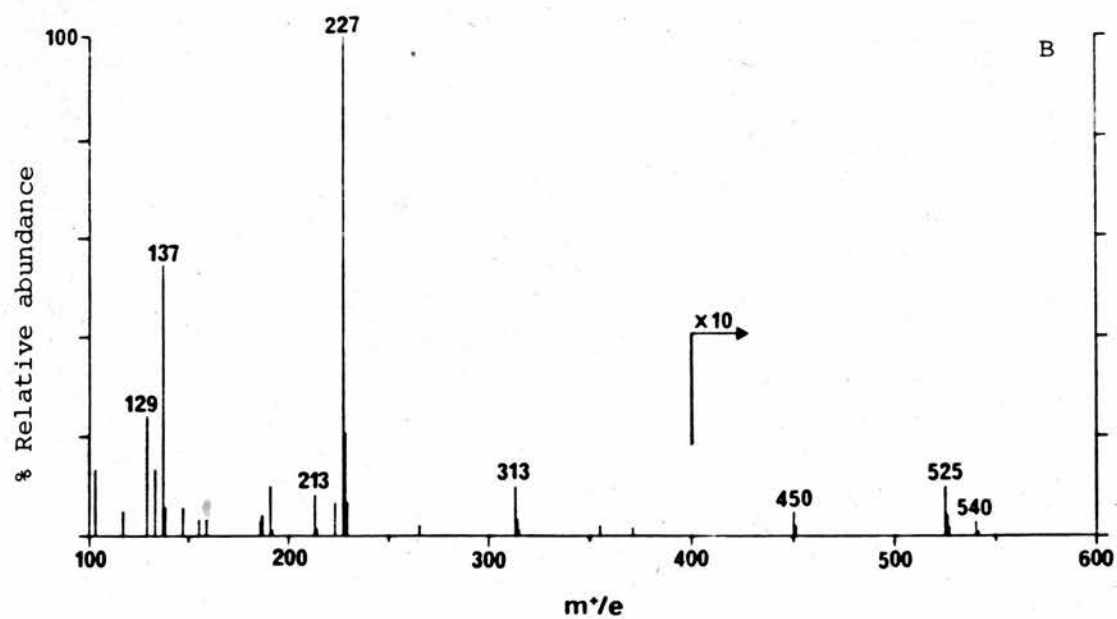
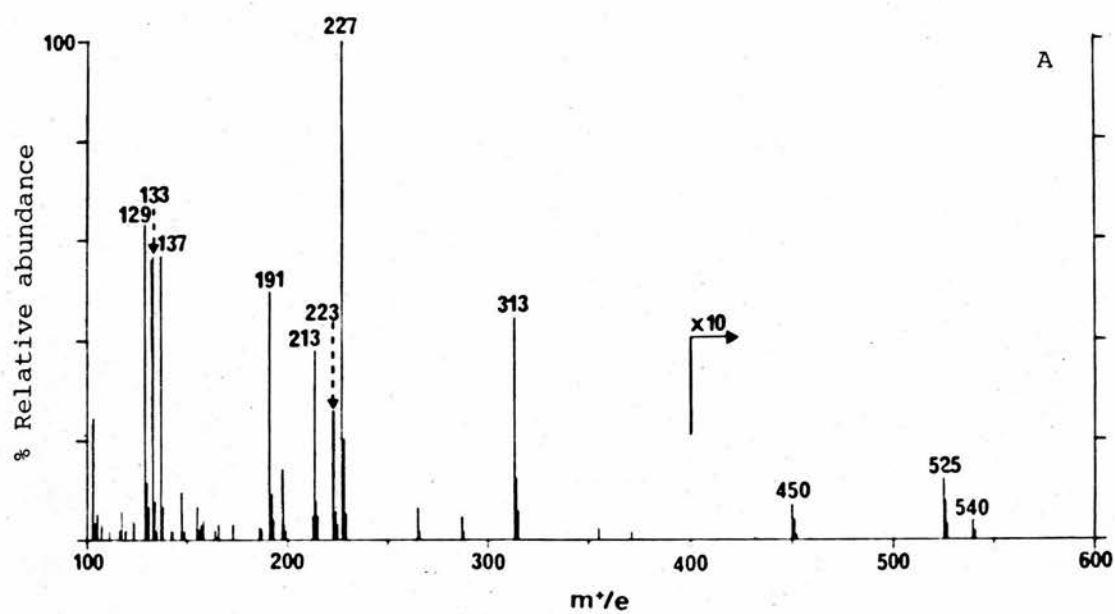


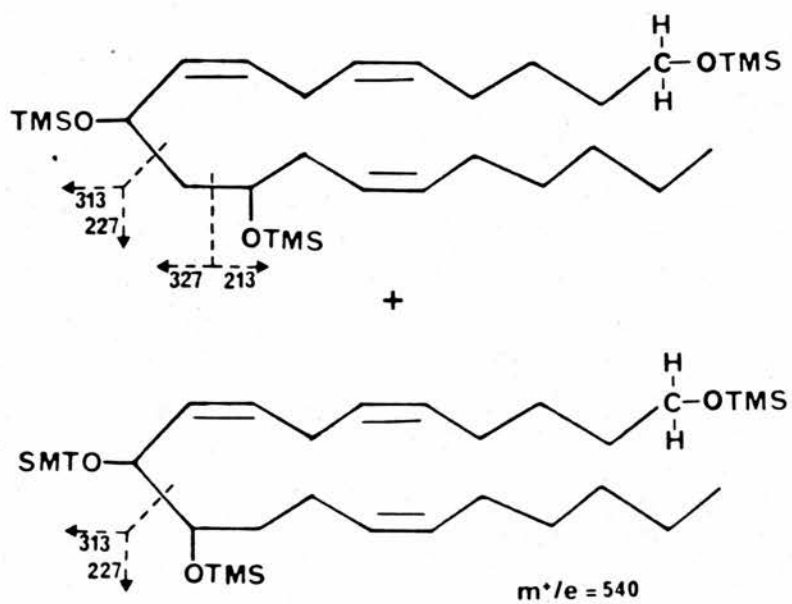
Figure 4.6 Reaction scheme for LiAlH_4 reduction

consistent with the products of the reaction scheme shown in Figure 4.6. The ratio of the relative abundance of the ion fragments at m^+/e 313/227 and m^+/e 227/213 altered in spectra taken at different positions on the GC peak (Figure 4.7) which suggested the presence of two slightly resolved components and indicated that the 1,3-product had a slightly shorter retention time than the 1,2-product. The GC-MS examination of the products of this reaction provided evidence for the presence of an epoxide group in the original material.

Gas chromatography of the n-butyl boronate/TMS derivative of the product of the LiAlH_4 reaction indicated that none of the TMS product described above remained and showed two partially resolved peaks (carbon values 23.8 and 24.2) with very similar mass spectra. These were consistent with the proposed products (Figure 4.6). The mass spectra contained a large number of low molecular weight ion fragments and in addition, diagnostic ion fragments at m^+/e 462 (molecular ion), 447, ($\text{M}-\text{CH}_3$), 378 ($\text{M}-\text{OBBu}$) and 372 ($\text{M}-\text{TMSOH}$). It was not possible to distinguish between the two GC components and therefore determine whether or not the two GC peaks corresponded to the two different products. This seemed a likely explanation. Since only 1,2- and 1,3-diols readily form boronates, the formation of the boronate derivative indicated that the hydroxyl group was adjacent to the epoxide in the original material and provided further evidence that the hydroxyl group was in the C-10 position.



- A. Mass spectrum recorded half way up GC peak
- B. Mass spectrum recorded at top of GC peak



(-TMSOH = 90)
 (-CH₃ = 15)

Figure 4.7 Mass spectra of EPHETA (Me/LiAlH₄/TMS)

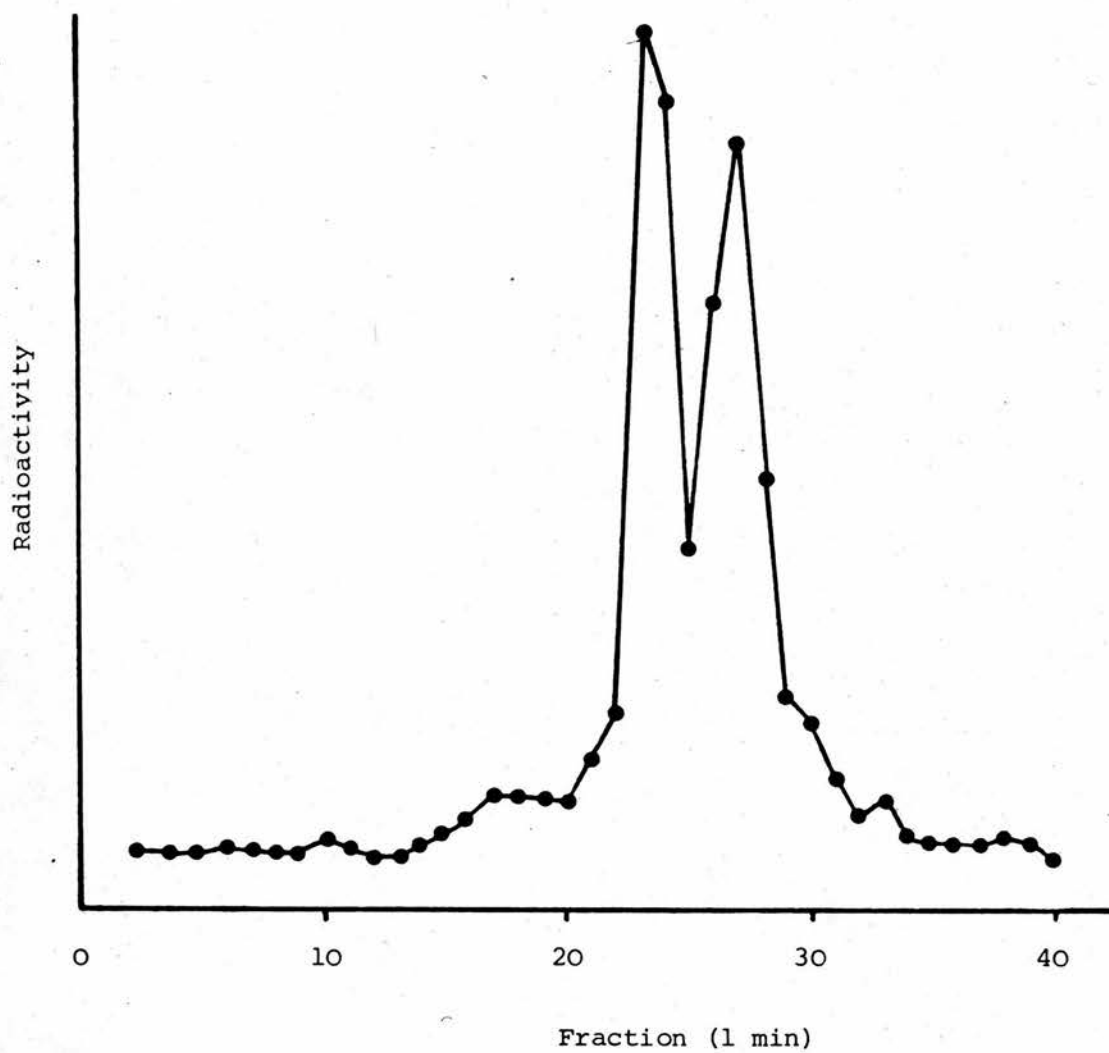


Figure 4.8 HPLC radiochromatogram of Zone II

High performance liquid chromatography (solvent system HPLC 2) of Zone II of the liquid-gel chromatographic separation showed two partially resolved peaks (Figure 4.8) which were shown to have identical carbon values on GC (22.05) as their Me/TMS derivatives and very similar mass spectra. These isomers had HPLC retention times between those of PGB₂ and 12-HETE (which were recorded on one occasion as: PGB₂ 30 min; EPHETA (A) 13 min; EPHETA (B) 15 min; 12-HETE 7.5 min). It was considered that the isomers resolved by HPLC were probably hydroxyl stereoisomers or geometrical epoxide isomers.

All the evidence outlined above indicated that this zone of the liquid-gel chromatographic separation contained isomers of 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (EPHETA) and not the proposed THETA intermediate, 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid. Further work is required to establish the configurations of the hydroxyl and epoxy groups. It was considered probable that the 5,6-, 8,9- and 14,15-double bonds had remained cis.

In the gas chromatogram of the Me/TMS of a horse platelet incubate, peak 2 (Figure 3.1) (carbon value 22.05) was shown to contain EPHETA. EPHETA was similarly identified in washed platelet incubates from human, dog, rabbit and cat. However, spectra obtained from these samples were not useful for determining the structure of EPHETA as ion fragments from even small amounts of the "12-HETE GC de-

composition product" were significant at the carbon value of EPHETA and unnecessarily complicated the structure elucidation of the then unidentified material. In these samples, selected ion monitoring of the ion fragments at m^+/e 269 (base peak of EPHETA, Me/TMS) and m^+/e 213 (principal ion fragment from the 12-HETE GC decomposition product Me/TMS) indicated that the single ion chromatograms of these ions had different GC profiles and were therefore derived from different components. This illustrated the necessity of the liquid-gel chromatographic clean-up of the platelet extract, prior to a GC-MS investigation of the structure of EPHETA, in order to remove these interfering components and hence simplify the interpretation of the mass spectra obtained.

Investigations using Epoxide Standards

While the structural elucidation work on EPHETA was in progress, a series of experiments was undertaken with the aim of determining the stability of an epoxide moiety under various conditions. These were necessary to establish reaction conditions for the LiAlH_4 reduction carried out on EPHETA, and to determine any potential effects of the standard derivatisation procedures employed. The results of these experiments are described in the remainder of this section.

An analogous model compound consisting of a straight chain fatty acid containing a hydroxyl group adjacent to an

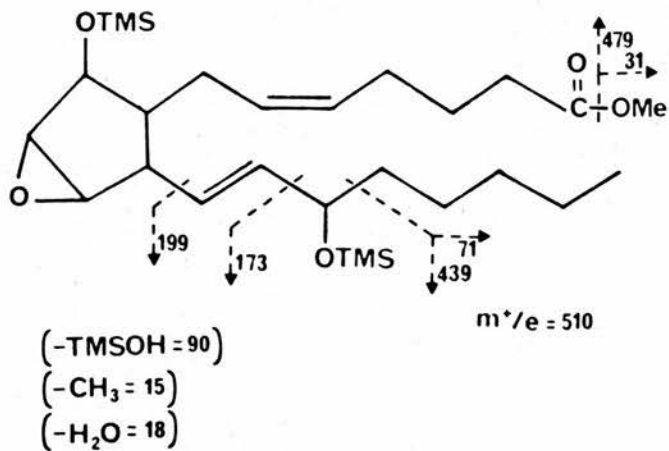
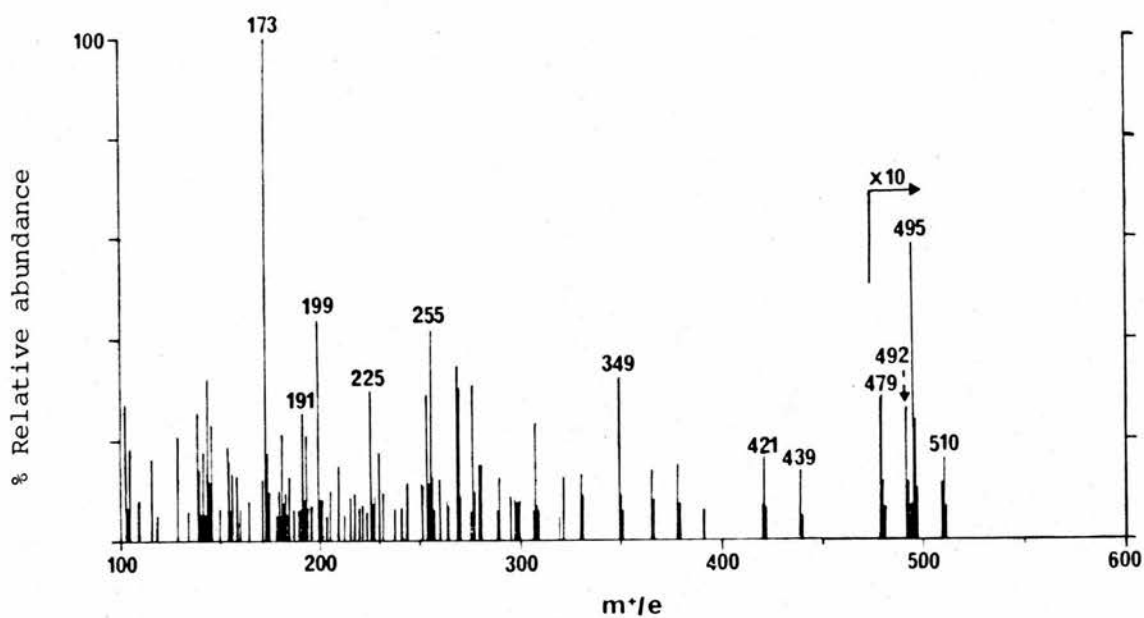
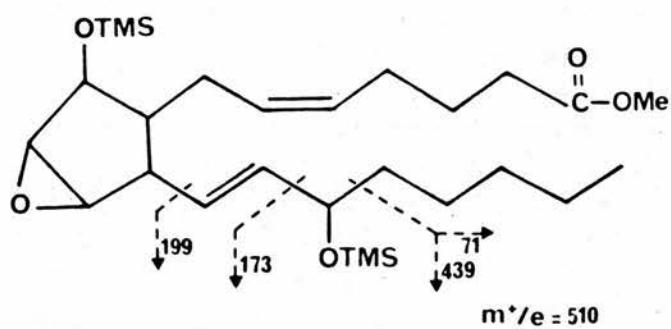
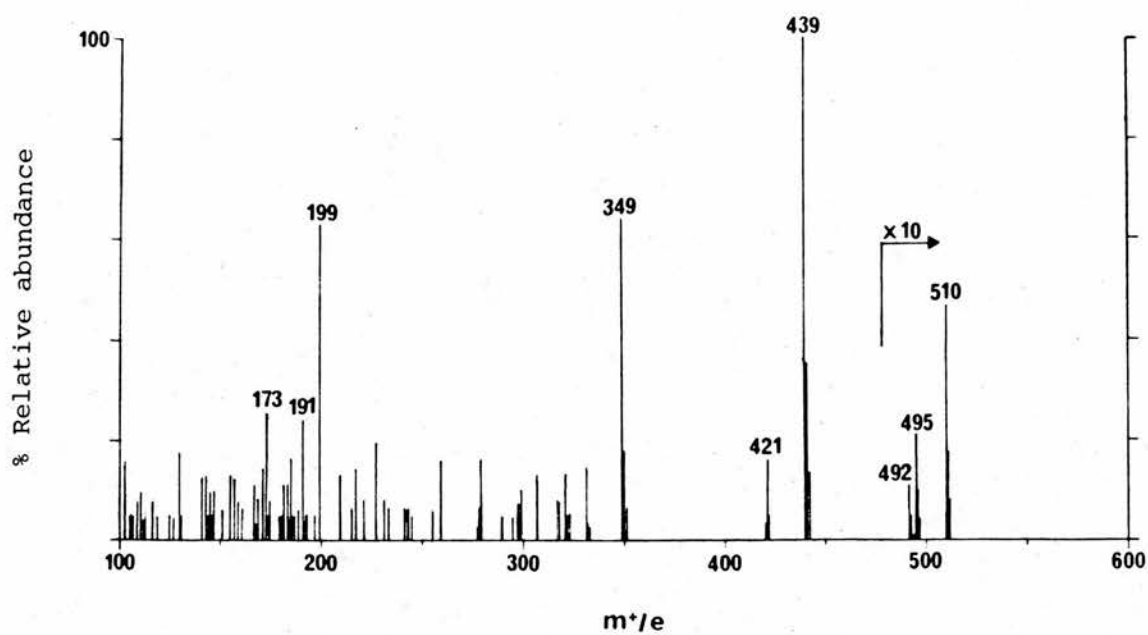


Figure 4.9 Mass spectrum of 10 α ,11 α -epoxy-11-deoxy-PGF₂ α (Me/TMS)



(-TMSOH = 90)
 (-CH₃ = 15)
 (-H₂O = 18)

Figure 4.10 Mass spectrum of 10 β ,11 β -epoxy-11-deoxy-PGF₂ α (Me/TMS)

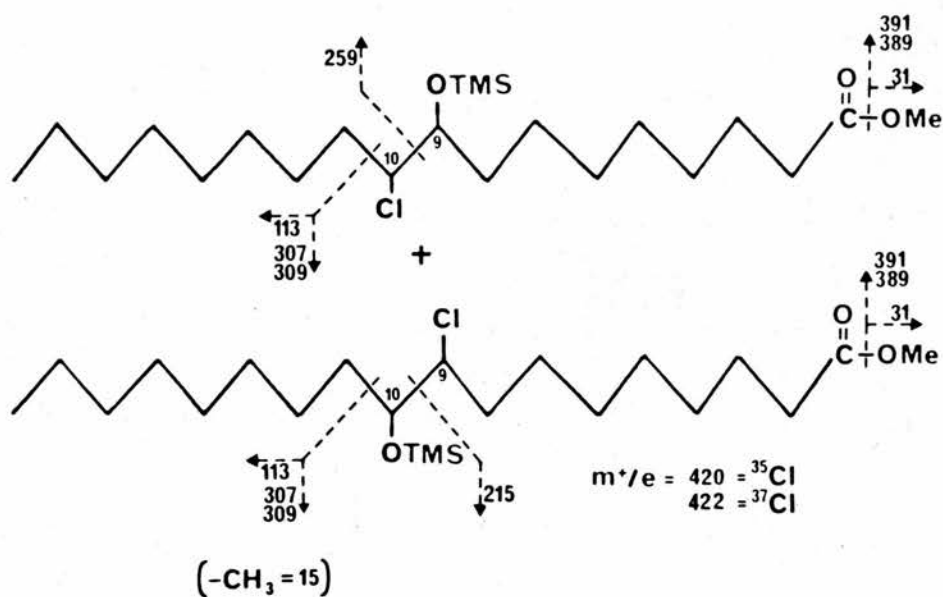
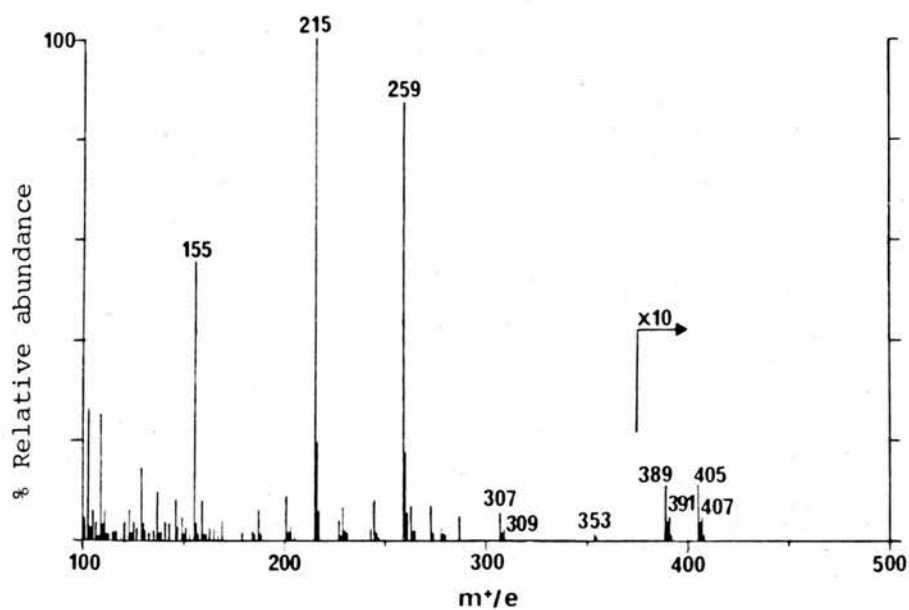


Figure 4.11 Mass spectrum of the product of the methyl ester of oleic acid epoxide after treatment with oxidizing reagents in pyridine and TMS ether formation

epoxide was desired but was not available. However, the methyl ester of oleic acid epoxide, 10 α ,11 α -epoxy-11-deoxy-PGF₂ α and the corresponding β -epoxy compound were available as standards.

It is noteworthy that epoxide moieties are well documented as being stable under gas chromatographic conditions (for example, McDonough and George, 1970; Emken, 1971, 1972; Gunstone and Jacobsberg, 1972). The three available standards were initially examined for any effects due to the methylation, silylation and hydrogenation procedures employed.

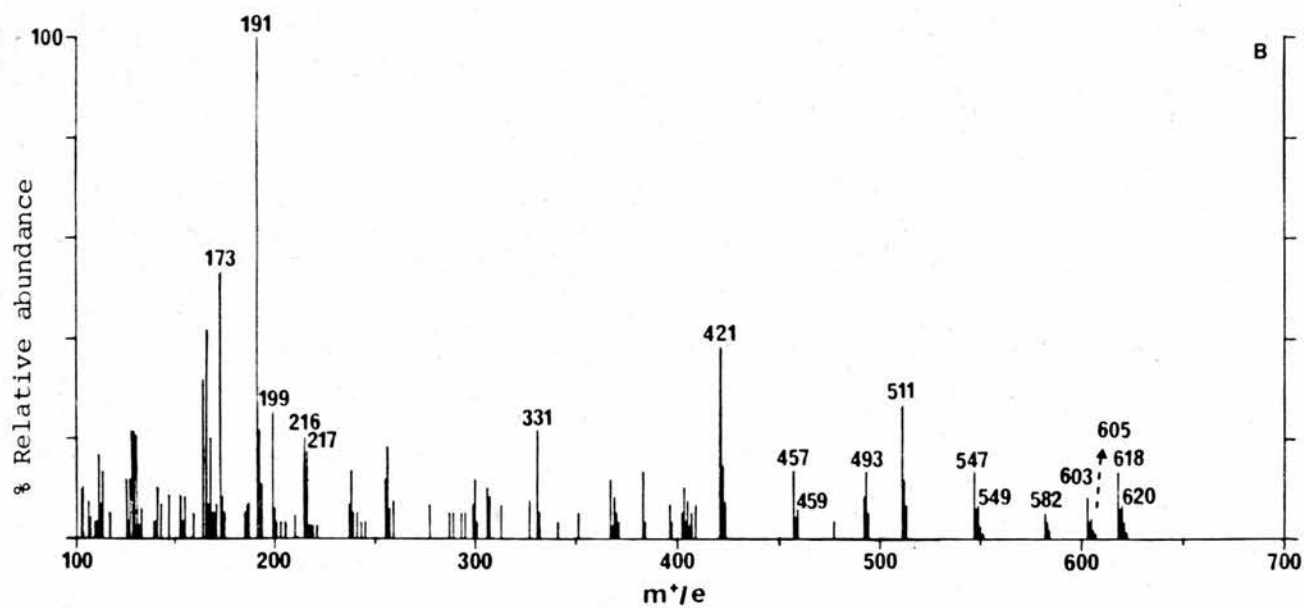
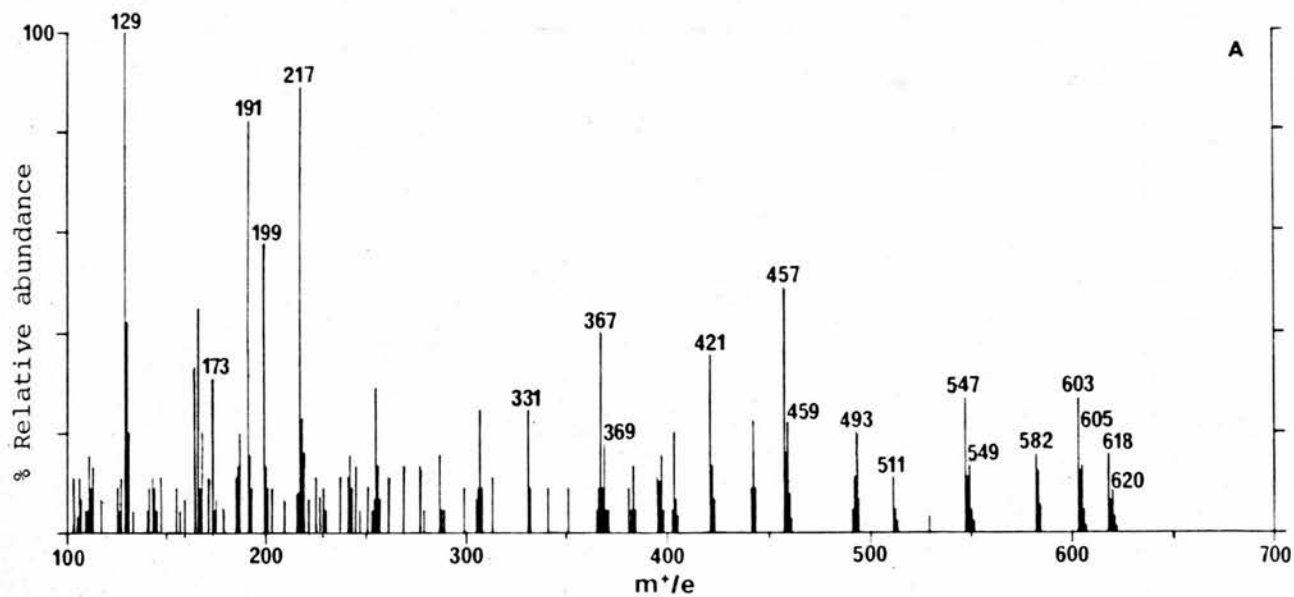
Gas chromatography of the methyl ester of oleic acid epoxide gave a single GC peak (carbon value 19.6) whose mass spectrum was very similar to that previously reported (Ryhage and Stenhagen, 1963). Treatment with BSTFA was shown to have no effect on the compound. GC-MS examination of the Me/TMS derivatives of the two prostaglandin analogues resulted in a single GC peak for each compound (carbon value α 24.0; β 24.3). Their mass spectra (Figure 4.9 and Figure 4.10 respectively) were interesting to compare as the relative abundance of the ion fragments in the different epoxide isomers varied considerably. The epoxide grouping in these compounds was clearly unaffected by the methylation and silylation procedures employed. It was also demonstrated that the methyl ester of oleic acid epoxide was unaffected by the hydrogenation procedure.

Potential effects of the oximating procedure were then investigated. When the methyl ester of oleic acid epoxide was subjected to the oximating procedure of reaction with methoxy- or butoxy-amine hydrochloride in pyridine, followed by TMS ether formation of the product (using BSTFA with or without 1% trimethylchlorosilane), the major GC peak did not correspond to the original epoxide and had a longer GC retention time (carbon value 21.6). This same major product was present whether methoxy- or butoxy-amine hydrochloride was used so it was concluded that the product did not contain the alkoxy group of the oximating reagent. The mass spectrum of this product (Figure 4.11) showed similarities with that obtained from 9,10-(bis TMS)-methyl stearate (carbon value 21.5) as both spectra contained their largest ion fragments at m^+/e 259 and 215. The carbon value of the "product of the oximation procedure" was 0.1 of a carbon value higher than that of methyl 9,10-bis(trimethylsilyloxy) stearate and a comparison of the mass spectra showed certain significant differences. A close examination of the mass spectrum of the unidentified product indicated the presence of the chlorine isotope pattern (^{35}Cl 75%, ^{37}Cl 25%) in various ion fragments and was concluded that the mass spectrum solved for a mixture of the two chloro-TMS compounds shown in Figure 4.11.

The reaction of an epoxide group with trimethylchlorosilane in pyridine was reported by Harvey et al (Harvey, Johnson and Horning, 1972; Harvey, Glazener, Stratton,

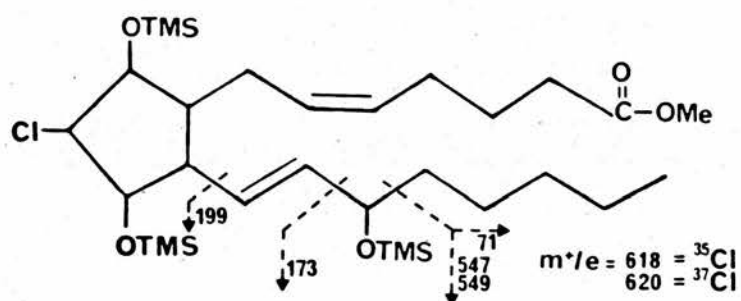
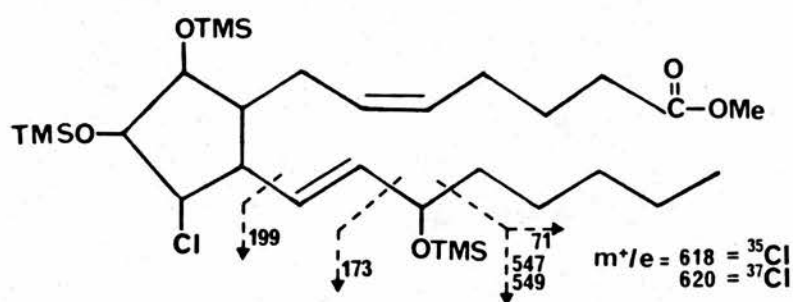
Johnson, Hill, Horning and Horning, 1972) to produce a TMS derivative of the chlorohydrin adduct. This reaction procedure was applied to the methyl ester of oleic acid epoxide (it is noteworthy that during this reaction, a copious white precipitate formed, presumably pyridine hydrochloride). Any additional hydroxyl groups in the molecule were then silylated by the addition of BSTFA to the reaction mixture (which also dissolved the white precipitate). GC-MS examination of this product indicated that the major GC peak had an identical carbon value and mass spectrum as the derivatised product resulting from the oximation reaction and thus confirmed the structure of the derivatised products of the oximation reaction as the two chloro-TMS compounds shown in Figure 4.11.

Oximation can also occur under acidic aqueous conditions (Jones, 1972). In the case of the methyl ester of oleic acid epoxide, this aqueous method of oximation resulted principally in the unchanged epoxide. Only relatively small amounts of the chloro-TMS compounds and in addition, small amounts of methyl 9,10-bis(trimethylsilyloxy) stearate were detected. Considerably smaller amounts of the epoxide were destroyed under these conditions and this procedure was considered more satisfactory than that using pyridine as solvent. The methyl ester of thromboxane B₂ was derivatised as a control and was found to oximate satisfactorily under both the acidic aqueous and basic organic conditions.



A. Mass spectrum recorded at carbon value 25.0

B. Mass spectrum recorded at carbon value 25.7



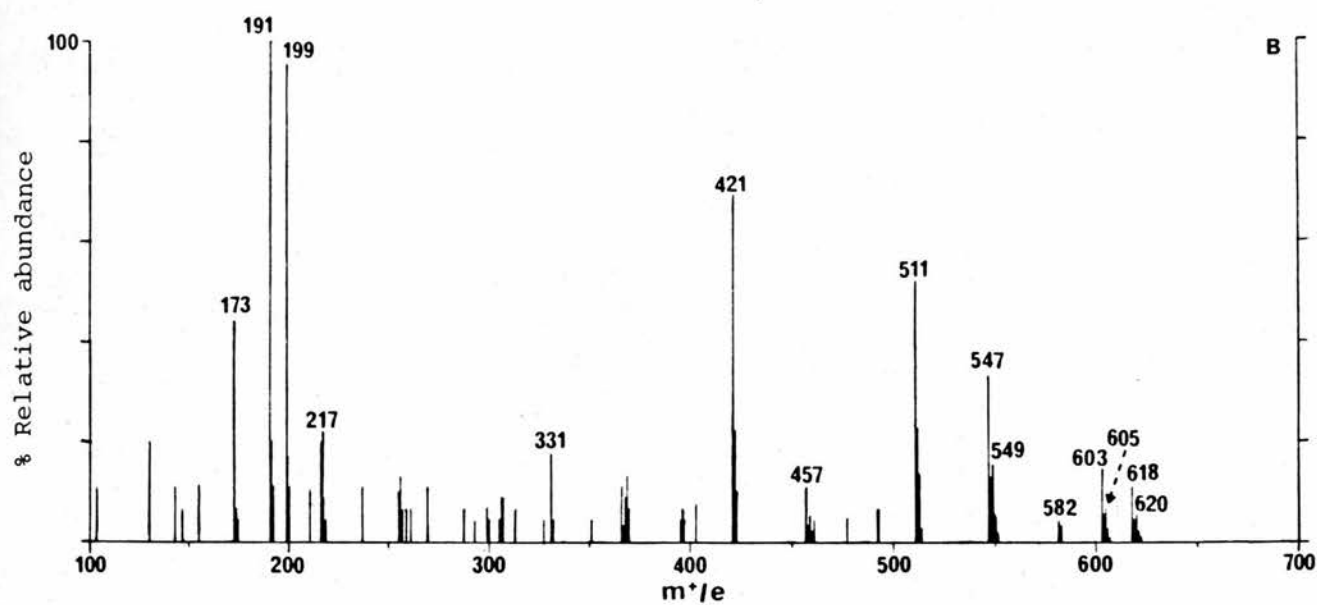
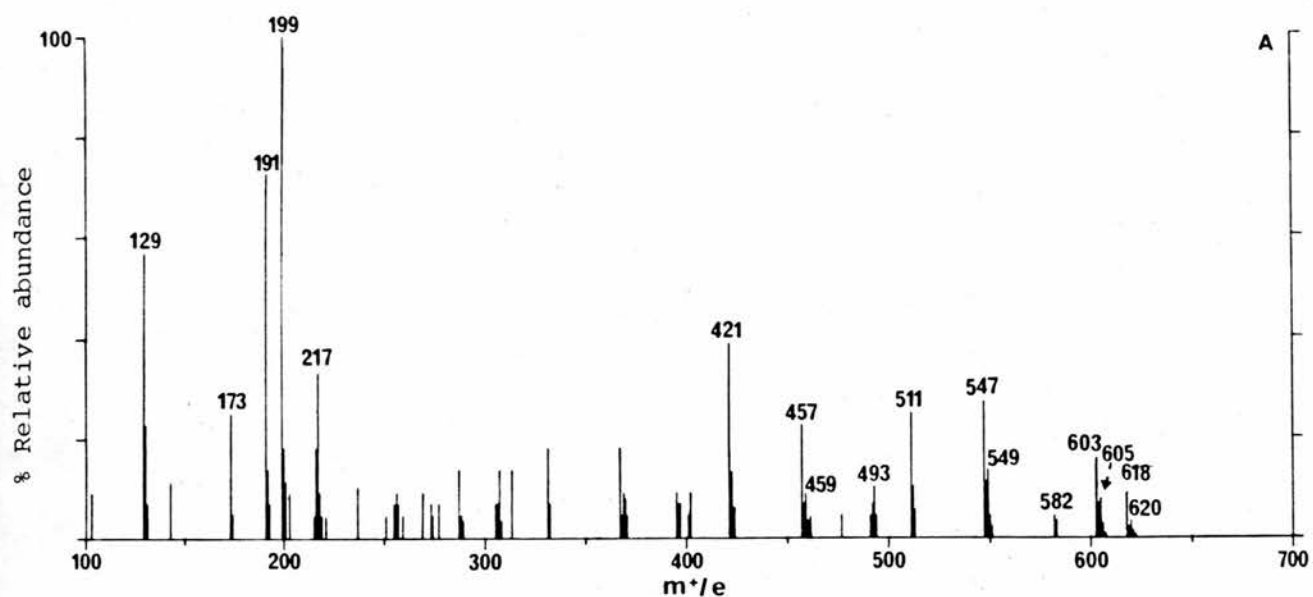
(-TMSOH = 90)
 (-CH₃ = 15)
 (-HCl = 36, 38)

Figure 4.12 Mass spectra of products of α -epoxide

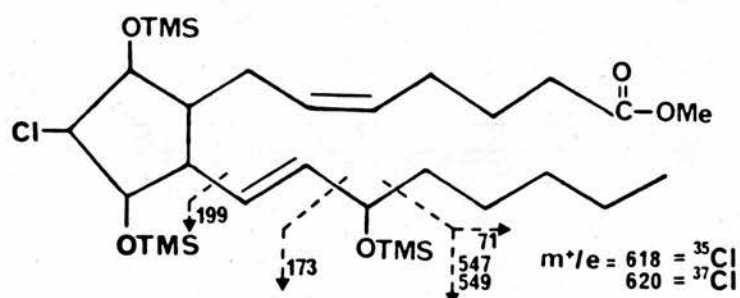
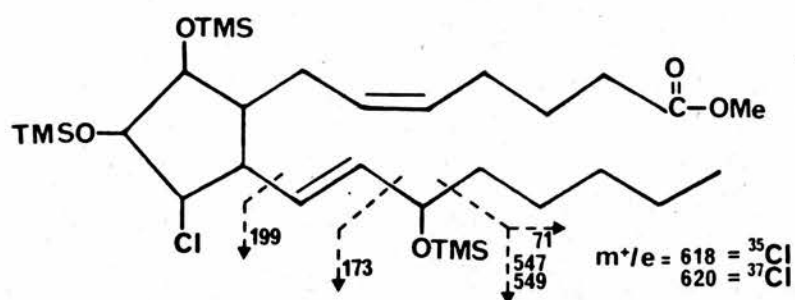
The effects of the pyridine oximating conditions on both the α - and β -epoxide prostaglandin analogues were then investigated. In contrast to the oleic epoxide standard, GC-MS examination of the products of both compounds after methylation, oximation and TMS ether formation indicated that the mass spectrum of the major GC peak for each product corresponded to that obtained previously for the Me/TMS derivative. In addition, two very small peaks (carbon values 25.0 and 25.7) were present in the chromatogram of the α -epoxide sample and a single small broad peak (carbon value 25.1-2) in the chromatogram of the β -epoxide sample. Larger amounts of these compounds were derivatised according to the same procedure in order to obtain spectra of these minor chromatographic peaks. The mass spectra of these minor components were found to be consistent with chloro-TMS products; the chlorine isotope pattern being present in several of the high mass ion fragments.

The two epoxides were also treated with trimethylchlorosilane/pyridine and BSTFA as described previously. The same products as the minor products of the oximation reaction were formed but in this case, they were the major chromatographic components and smaller amounts of the Me/TMS derivatives were present.

In the case of the α -epoxide, the mass spectra of the two GC peaks (carbon values 25.0 and 25.7) were similar (Figure 4.12) although the relative intensity of the



- A. Mass spectrum recorded at carbon value 25.1
 B. Mass spectrum recorded at carbon value 25.2



(-TMSOH = 90)
 (-CH₃ = 15)
 (-HCl = 36, 38)

Figure 4.13 Mass spectra of products of β -epoxide

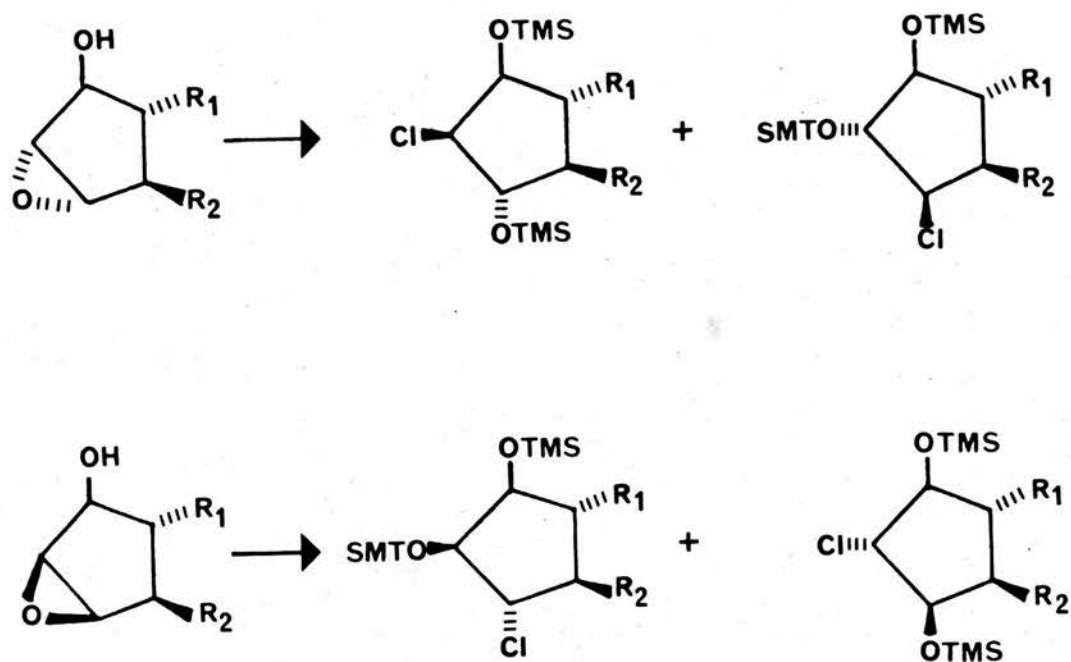
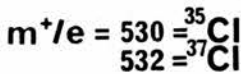


Figure 4.14 Proposed reaction products from α - and β -epoxide analogues

different ion fragments differed. The peak shape of the product of the β -epoxide suggested two partially resolved components (carbon values 25.1 and 25.2); the mass spectra of which (Figure 4.13) were very similar, again differing only in the relative intensity of the different ion fragments. The most likely explanation of these observations is that the two products formed from these reactions (Figure 4.14) completely resolved in the case of the α -epoxide but only partially resolved in the case of the β -epoxide. It is noteworthy that in the mass spectra the chlorine isotope effect is affected by an isotope effect from the other atoms in the ion fragments.

These experiments indicated that the ease of opening of the epoxide ring in oleic epoxide and the prostaglandin analogues differed considerably and demonstrated that the ease of opening of an epoxide moiety depended on its chemical environment. The need for caution in applying standard derivatisation procedures to certain epoxides was apparent.

In the case of EPHETA, the molecule was found to be unaffected by the pyridine oximation procedure, although if comparable amounts of the chloro-TMS products had been present as those formed in the case of the α - and β -epoxy prostaglandin analogues, these would not have been detected. GC-MS examination of the product after treatment of EPHETA with trimethylchlorosilane / pyridine and BSTFA



silane, pyridine/BSTFA)

indicated that some of the Me/TMS derivative (carbon value 22.05) was present but it was not the major component. The major chromatographic component was composed of a principal peak (carbon value 24.1) which was partially resolved from a considerably smaller peak (carbon value 24.4). The mass spectrum of the principal peak (carbon value 24.1) had a base peak at m^+/e 269 (compare the mass spectrum of the Me/TMS derivative, Figure 4.3) and although the most intense ions in the spectrum corresponded to non-chlorine TMS fragments, the distinctive chlorine isotope pattern was present in very small ion fragments at m^+/e 532, 530 (molecular ion), 517, 515 ($M-CH_3$) and in more intense ion fragments at 263, 261 ($M-269$) (Figure 4.15). The spectra obtained from this derivative were not as definitive as hoped, but provided additional evidence for the presence of an epoxide group in EPHETA.

Although clearly not the ideal compound, the methyl ester of oleic acid epoxide was used as a standard to determine the experimental conditions for the $LiAlH_4$ reduction and to ascertain whether or not this reaction would be feasible on the small amounts of EPHETA which were available (in the order of 250 μg). Initial experiments involved heating the reaction mixture for 18 h and 72 h at 60°C. GC-MS examination of the TMS derivatives of the products indicated that while starting material was present in the 18 h sample, none remained in the 72 h sample. Two partially resolved chromatographic peaks (Figure 4.16) were

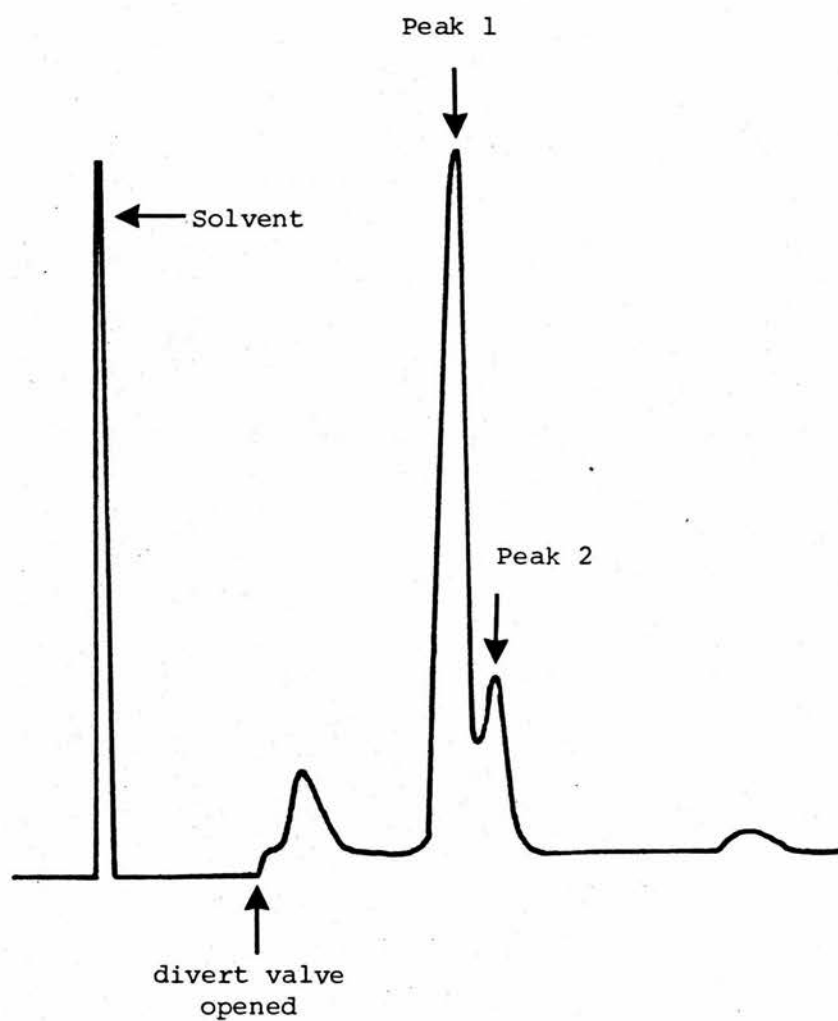


Figure 4.16 Gas chromatogram of the TMS ether of the product of the methyl ester of oleic acid epoxide following treatment with LiAlH_4 for 72 h

Compound

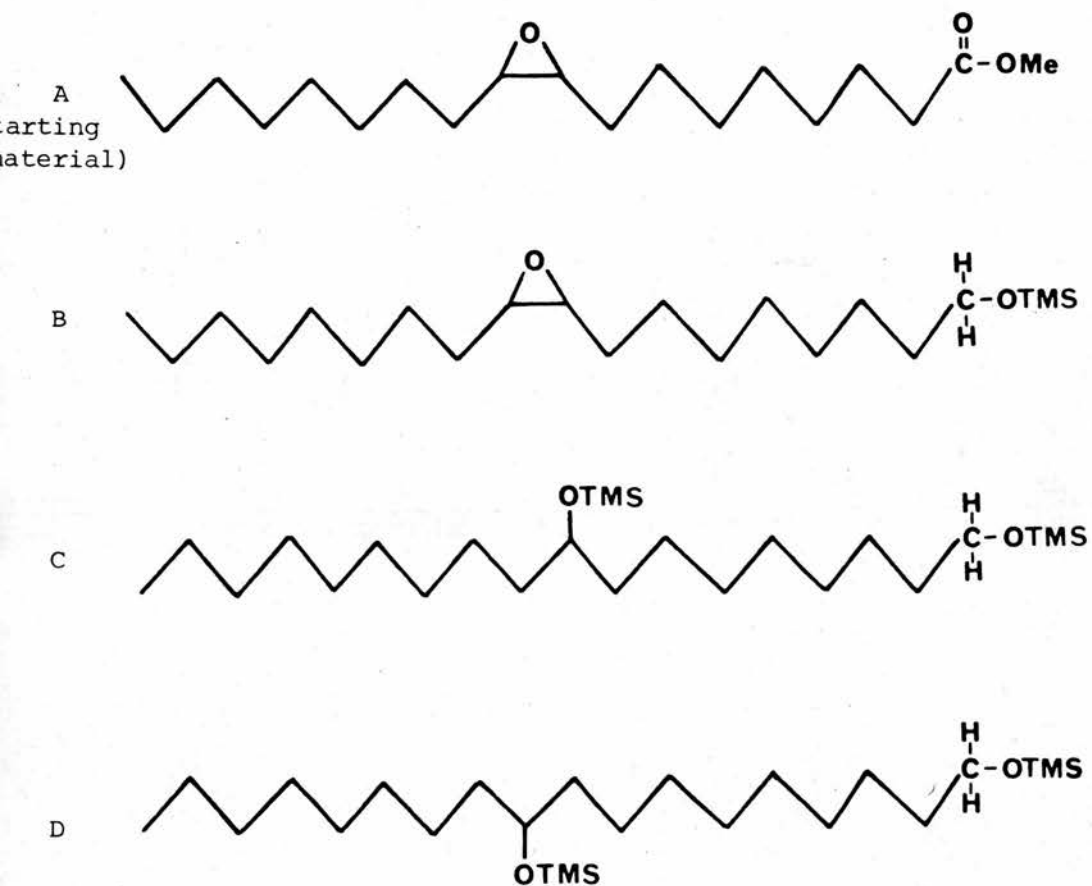


Figure 4.17 Possible products of LiAlH_4 reduction

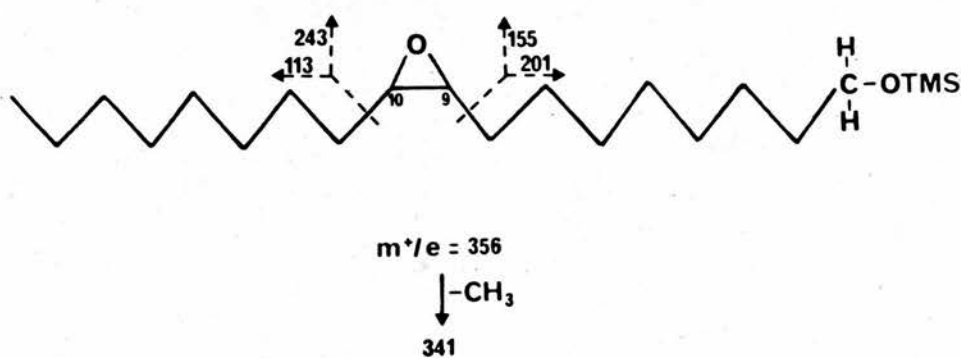
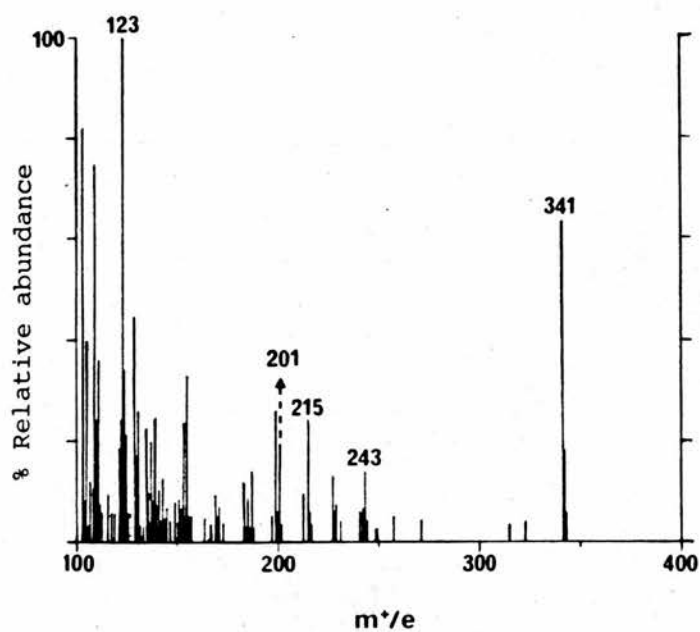


Figure 4.18a Mass spectrum of Peak 1, Figure 4.16

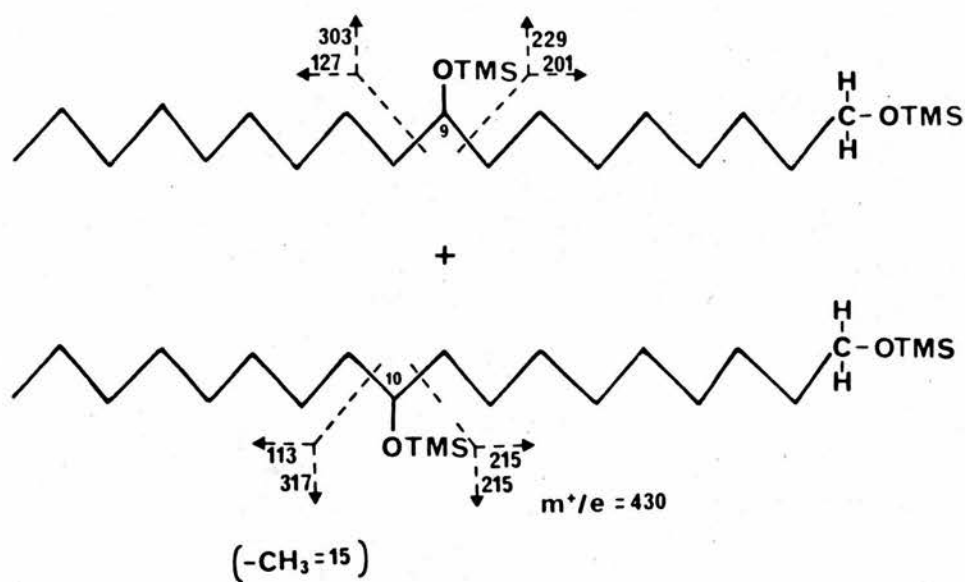
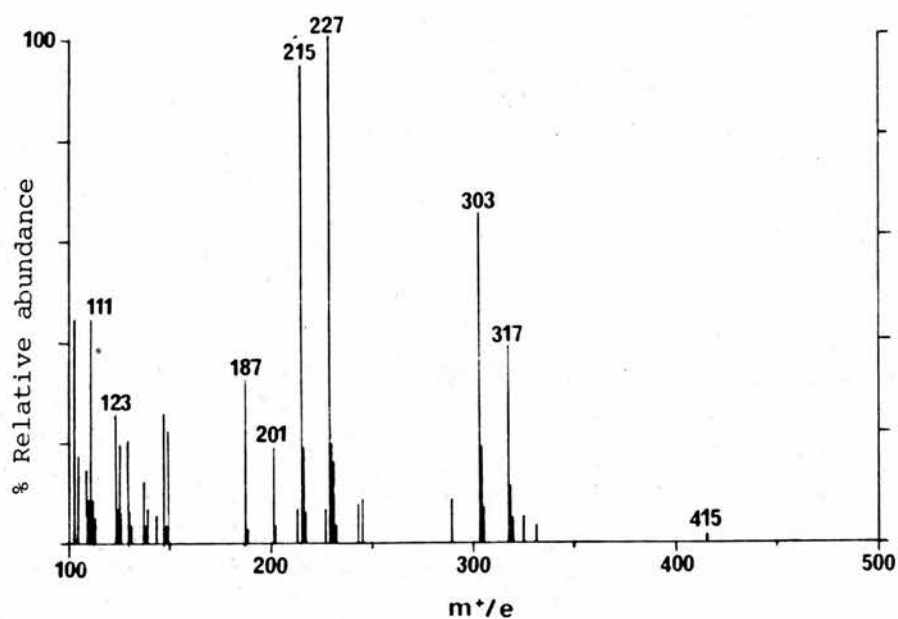


Figure 4.18b Mass spectrum of Peak 2, Figure 4.16

present in the 72 h sample (carbon value 20.0 and 20.3), the longer retained peak corresponding to the minor product. The possible products are shown in Figure 4.17. The mass spectra of the two GC peaks (Figure 4.18) indicated that the major peak (peak 1, Figure 4.16) corresponded to compound B (Figure 4.17) and that compounds C and D (Figure 4.17), the required products, were present in the minor peak (peak 2, Figure 4.16). An intense ion fragment corresponding to the loss of a methyl group from the molecular ion was reported to be characteristic for TMS derivatives of alcohols (Budzikiewicz, Djerassi and Williams, 1967a) (Figure 4.18a).

This reaction was repeated, on two subsequent occasions, with heating for 72 h at 60°C using a fresh supply of LiAlH_4 . On each occasion, GC-MS examination of the TMS derivatives of the products indicated that the reactions had gone to completion, as only a single chromatographic peak was detected whose carbon value (20.3) and mass spectrum corresponded to the minor product detected previously. The mass spectrum was consistent with a mixture of compounds C and D, the products expected from this LiAlH_4 reduction. These products would not be expected to resolve on the GC. This same experimental procedure was applied to provide evidence for the presence of an epoxide group in EPHETA as described earlier.

Zone III of the Liquid-Gel Chromatographic Separation

GC-MS examination of the Me/TMS derivative of Zone III of the liquid-gel chromatographic separation of the platelet incubate (Figure 3.6) indicated a single major GC peak (carbon value 19.3). The mass spectrum was consistent with a C17 fatty acid methyl ester containing three double bonds and one TMS group and was in agreement with that reported for the Me/TMS derivative of the C17 hydroxy acid HHT (Hamberg and Samuelsson, 1974a).

A comparison of the amounts of HHT present as indicated by scintillation counting and GC-MS did not correlate satisfactorily and indicated that possibly another component was present in this zone from the liquid-gel chromatographic separation. The formation of HHT is inhibited by indomethacin as it is a product of the prostaglandin cyclo-oxygenase pathway. A radioactive peak was still present in this zone of the liquid-gel chromatogram when indomethacin was present in the platelet incubate. GC-MS examination of the Me/TMS derivative of this incubate confirmed that the HHT biosynthesis had been virtually completely inhibited, as only trace amounts of HHT were detected. There were not, however, any other significant chromatographic peaks except that

attributable to the indomethacin derivative which was found to chromatograph in this zone of the liquid-gel column. The indomethacin derivative (methyl ester) possessed a higher carbon value (25.6) than previously detected arachidonic acid metabolites and therefore the potential gas chromatographic interference of the indomethacin derivative was discounted.

After methylation followed by oximation (using methoxy- and butoxy-amine hydrochloride) and TMS ether formation or treatment of the material with trimethylchlorosilane/pyridine and BSTFA (in attempts to detect a chromatographically unstable epoxide) of samples of zone III (both from incubates with and without indomethacin present), no additional product was detected by GC-MS. This indicated that this product had not been stabilised for gas chromatography by the derivatisation procedures employed. In addition, GC-MS examination of the Me/TMS derivative of Zone III after further incubation with washed human platelets did not result in the detection of any additional compound. This indicated that this zone did not contain a compound which was an intermediate in the formation of any of the previously mentioned compounds. Further work is required to identify this product(s) of the platelet incubation which is not formed via the prostaglandin cyclo-oxygenase pathway.

In order to check that this additional product was not an auto-oxidation product of arachidonic acid, arachidonic

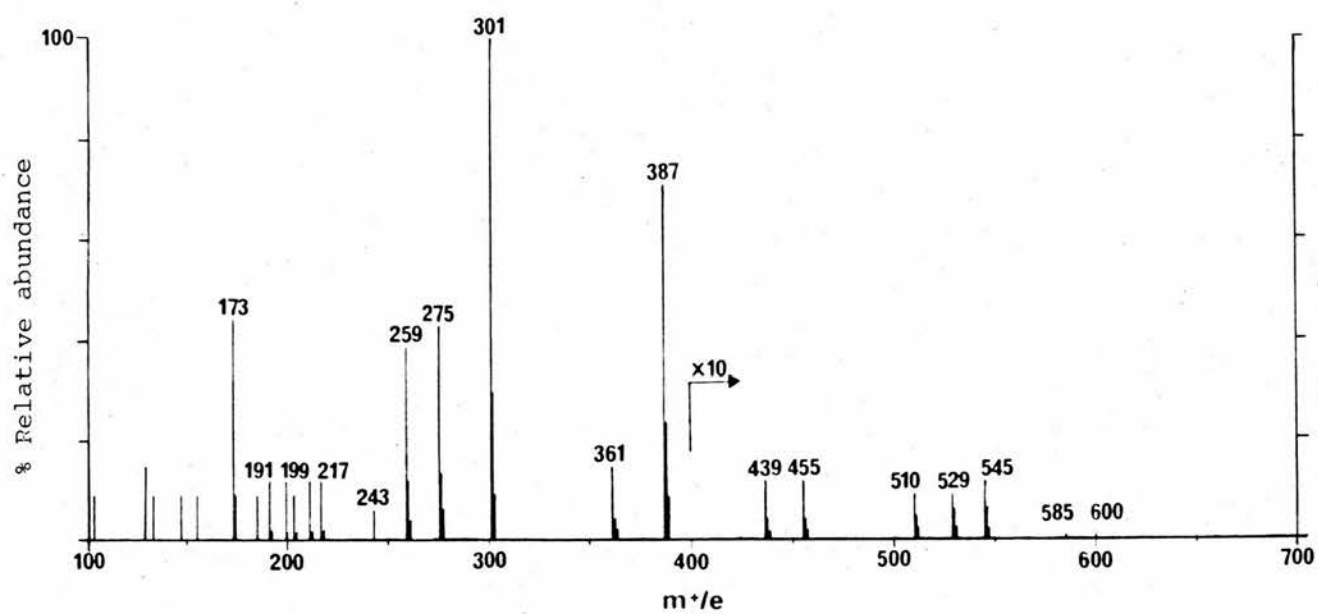


Figure 4.19 Mass spectrum of unidentified material (Me/TMS)

acid (containing 1-¹⁴C-arachidonic acid as tracer) was heated at 37°C for 30 min in Krebs-Henseleit buffer and extracted in the same manner as the platelet incubates. The extract was then subjected to liquid-gel chromatography. The resultant chromatogram indicated that there was no radioactivity in this zone from the chromatographic separation and demonstrated that the unidentified material was a product formed as a result of the presence of platelets in the incubations.

Zone VI of the Liquid-Gel Chromatographic Separation

On one occasion, very small amounts of radioactivity were detected in fractions of the liquid-gel chromatographic separation (Zone VI, Figure 3.6) which eluted later than those containing 8,11,12-THETA and thromboxane B₂. GC-MS examination of the Me/TMS derivative of these fractions showed a single GC peak (carbon value 24.3) whose mass spectrum (Figure 4.19) indicated a compound of molecular weight m^+/e 600. Comparison of the spectra of the Me/TMS and Et/TMS derivatives demonstrated which ions in the Me/TMS spectrum contained the ester group and indicated that the base peak in the Me/TMS spectrum at m^+/e 301 and the large ion fragment at m^+/e 275 did not contain the ester moiety, whereas the large ion fragments at m^+/e 387 (M-213) and m^+/e 259 did. Difficulty was encountered in attempting to rationalise the large ion fragments at m^+/e 301 and 387 as resulting from the same molecule. The

spectra contained certain characteristic "prostaglandin-like" ions. For example, the ions at m^+/e 173 which would normally indicate a TMS group in the C-15 position; m^+/e 199 which would be attributable to the ω side chain; m^+/e 217 which was indicative of a 1,3 di-TMS function; m^+/e 191 the base peak of $\text{PGF}_{2\alpha}$ (Me/TMS) which is this prostaglandin derivative is attributable to the ion fragment ($\text{C}_8\text{H}_6 \text{ OTMS}$). The material did not form an oxime which indicated that no keto group or hemi-acetal moiety was present in the molecule. The mass spectra of this compound(s) were not solved since further material did not become available and therefore further structural elucidation was not possible.

Investigation by GC-MS of the radioactive zones of the liquid-gel chromatographic separation, other than those described in Section 3, resulted in the detection of a compound analogous to the proposed THETA intermediate (8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid). The formation of this analogous compound, like THETA, was not inhibited by indomethacin but was inhibited by ETYA. Examination of a number of derivatives indicated its structure as 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (EPHETA) and not the proposed THETA intermediate. Isomers of EPHETA were resolved by high performance liquid chromatography.

A possible mechanism suggested for the formation of EPHETA involved further transformation of 12-HPETE; analogous conversions of C18 fatty acid hydroperoxides have been reported (Gardner, Kleiman and Weisleder, 1974; Hamberg, 1975).

Non-allylic epoxy-hydroxy acids are considerably more stable towards nucleophiles than allylic epoxy-hydroxy acids. 11-Hydroxy-12,13-epoxy-9-octadecenoic acid (a non-allylic epoxy-hydroxy acid) is stable in aqueous ethanol at 100°C for at least 3 hours (Hamberg and Gotthammar, 1973) whereas under the same conditions, 9-hydroxy-12,13-epoxy-10-octadenoic acid (an allylic epoxy-hydroxy acid)

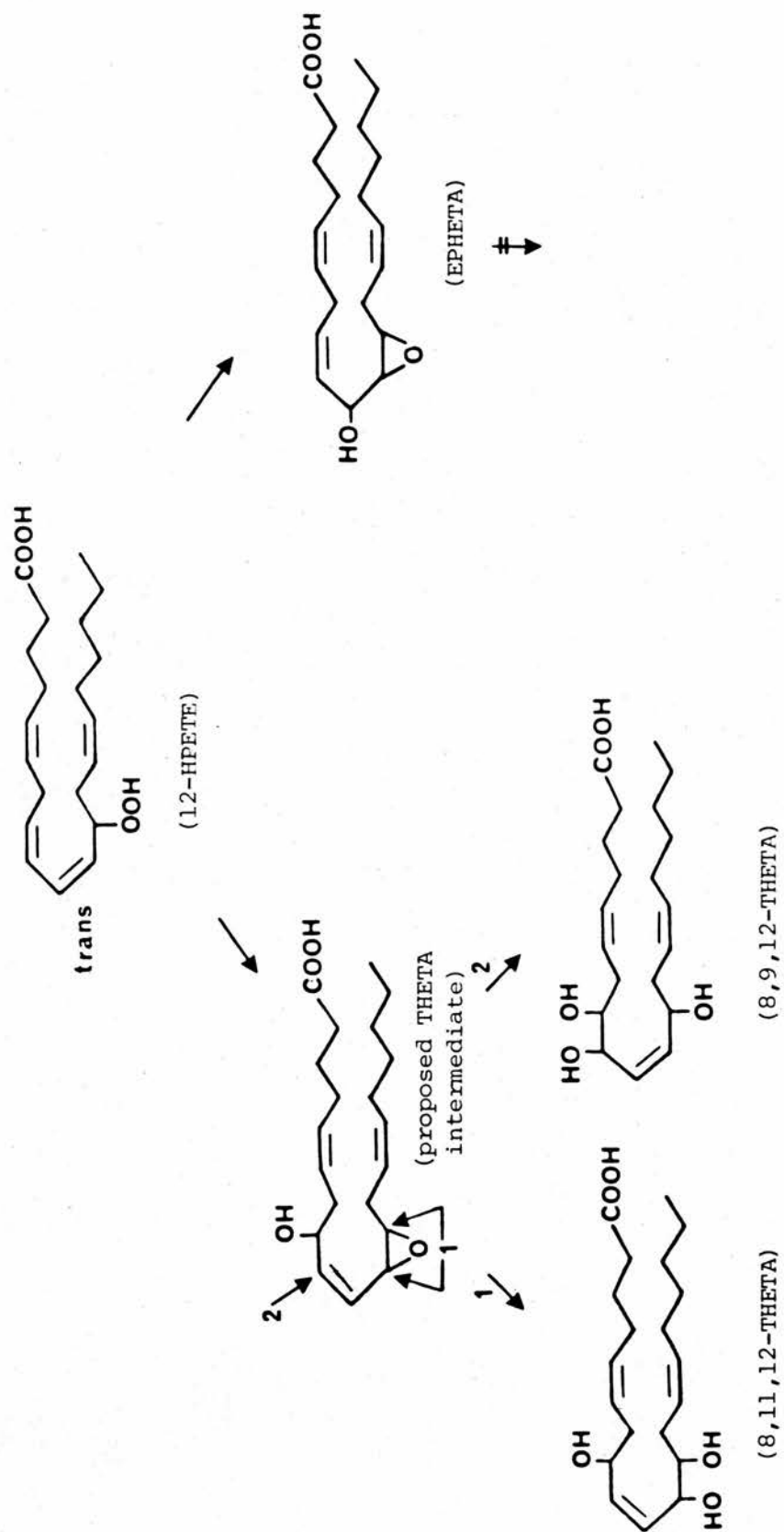


Figure 4.20 Proposed reaction scheme for formation of THETA and EPHETA

is converted into a mixture of 9,10,13-trihydroxy-11-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid (Hamberg, personal communication). It would therefore seem probable that both 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (EPHETA) and the proposed THETA intermediate, 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid form from 12-HPETE (compare Hamberg, 1975). Only the former more stable non-allylic epoxy-hydroxy acid accumulated and was detected whereas the latter, less stable allylic epoxy-hydroxy compound was converted to THETA by the addition of water (Figure 4.20) and was therefore not detected. It is noteworthy that solutions of EPHETA stored at -20°C in methanol were stable for several months. Attempts to detect the proposed THETA intermediate by shortening the incubation period were not successful.

The product of the platelet incubate which co-chromatographed with the C17 monohydroxy acid HHT has not been identified and further work is required to determine the structure of this material. Maclouf et al (Maclouf, Bernard, Rigaud, Rocquet and Breton, 1977) detected a similar product on incubating arachidonic acid with spleen microsomes from whole body irradiated rats. The formation of their product, which co-chromatographed on TLC with HHT, was similarly not inhibited by indomethacin. They did not identify their product.

It was of interest whether or not these compounds possessed any pharmacological activity and a number of tests were performed which are described in Section 5 of this thesis.

SECTION 5

THE PHARMACOLOGICAL TESTING OF THE NEWLY
IDENTIFIED ARACHIDONIC ACID METABOLITES

The newly identified arachidonic acid metabolites were tested in "prostaglandin-sensitive" biological assay systems in order to determine whether or not they possessed similar biological activity to the prostaglandins. These tests included their effects on platelet aggregation (either pro-aggregatory or anti-aggregatory with respect to aggregation induced by arachidonic acid and ADP), cat blood pressure and their ability to contract strips of rabbit aorta, rat fundus and guinea pig ileum.

In addition, the effects of these compounds on enzymes involved in prostaglandin synthesis (prostaglandin cyclooxygenase, thromboxane synthetase and PGI₂ synthetase) were determined. The effects on the first two enzymes were determined from the platelet aggregation studies and the effect on PGI₂ synthetase from experiments involving incubation of arachidonic acid with aortic rings.

The chemotactic activity of each of the compounds was also determined in particular because the lipxygenase product 12-HETE had been reported to possess this type of activity. In addition, there were reports (at times conflicting) concerning the activity of several prostaglandins, thromboxane B₂ and HHT. These reports are fully referenced later in this section.

Quantification of the arachidonic acid metabolites tested in these studies were determined on small amounts of each sample, both by liquid scintillation counting and by comparison of the peak area of the Me/TMS derivative on GC-MS with standard C22 and C24 fatty acid methyl esters. Good correlation (less than 20% variation) was obtained for 8,11,12-THETA (samples containing less than 10% thromboxane B₂, which was accounted for in the quantification, were chosen for testing), 8,9,12-THETA and EPHETA. Zone III was quantified only by scintillation counting - an estimation of the amounts of HHT present by GC-MS indicated that only 25-40% of the radioactivity in Zone III was accounted for by the HHT. It was considered that any pronounced biological activity of the additional unidentified material (section 4.3) would be detected, as HHT itself has not been reported to have activity other than being chemotactic at high doses (Goetzl and Gorman, 1978).

The chemotaxis work described in this section was performed with Dr. P. J. Kerry. The experiments on the cats were carried out with Dr. R. L. Jones.

1. Platelet Aggregation

In the case of prostaglandins, the platelet aggregation assay system is sensitive to the anti-aggregatory substances PGI_2 , PGD_2 and PGE_1 . The reader is referred to a paper by Moncada et al (Moncada, Vane and Whittle, 1977) for a comparison of the relative potency of these inhibitors of platelet aggregation in several species. The assay is also sensitive to the prostaglandin endoperoxides (Hamberg, Svensson, Wakabayshi and Samuelsson, 1974) and the highly unstable thromboxane A_2 (Hamberg, Svensson and Samuelsson, 1975) which induce irreversible aggregation.

8,11,12-THETA, EPHETA and Zone III from the liquid-gel chromatographic separation (at a concentration of $10 \mu\text{g}.\text{ml}^{-1}$) had neither pro-aggregatory effects nor anti-aggregatory effects (with respect to aggregation induced with arachidonic acid or ADP). However, a partial inhibitory effect of the 8,9,12-THETA solution ($10 \mu\text{g}.\text{ml}^{-1}$) to aggregation induced with either arachidonic acid or ADP was detected. The response was equivalent to that caused by 12.5 ng of PGD_2 , a possible contaminant.

PGD₂ is unstable under basic conditions and a sample of the 8,9,12-THETA solution was treated with base to determine the likelihood of contamination. Basic conditions (which were demonstrated to abolish the effects of a PGD₂ standard on platelet aggregation) resulted in the loss of anti-aggregatory activity of the 8,9,12-THETA solution without loss of 8,9,12-THETA as indicated by GC-MS analysis. The presence of PGD₂ was not, however, confirmed by GC-MS. Attempts, which involved preparing the remainder of the sample (approximately 10 µg 8,9,12-THETA) as the Me/BuO/TMS derivative and selected ion monitoring, indicated that the derivatised sample was not sufficiently clean to satisfactorily determine whether or not the very small amounts of PGD₂ indicated by the platelet experiments were present. PGD₂ would be expected to elute from the liquid-gel column with a similar retention to 8,9,12-THETA and it was deduced that the anti-aggregatory activity observed in the 8,9,12-THETA solution was almost certainly attributable to the presence of small amounts of PGD₂.

It was concluded that none of the compounds had any significant effect on the platelet aggregation response.

2. Prostaglandin I₂ Production

It was of interest to determine if any of the compounds affected the prostaglandin cyclo-oxygenase, thromboxane synthetase or PGI₂ synthetase enzymes. If any of the compounds had an inhibitory effect on either the prostaglandin cyclo-oxygenase or thromboxane synthetase, this would have resulted in an effect on the platelet aggregation response to arachidonic acid (Section 5.2.1). It was therefore concluded that none of the novel arachidonic acid metabolites had an effect on these enzymes at the concentrations tested.

In order to determine their effect on PGI₂ biosynthesis, a biological assay method, as opposed to a GC-MS assay method, was developed as it was considered that the biological assay was simpler to develop and use, and would provide a faster indication of any inhibitory effect of the compounds. It was intended to develop a more precise GC-MS assay should the biological assay indicate that any of the metabolites possessed inhibitory action.

The biological assay method involved incubation of aortic rings with arachidonic acid to produce PGI₂, transfer of a portion of the incubate to a platelet suspension and monitoring its effect on the aggre-

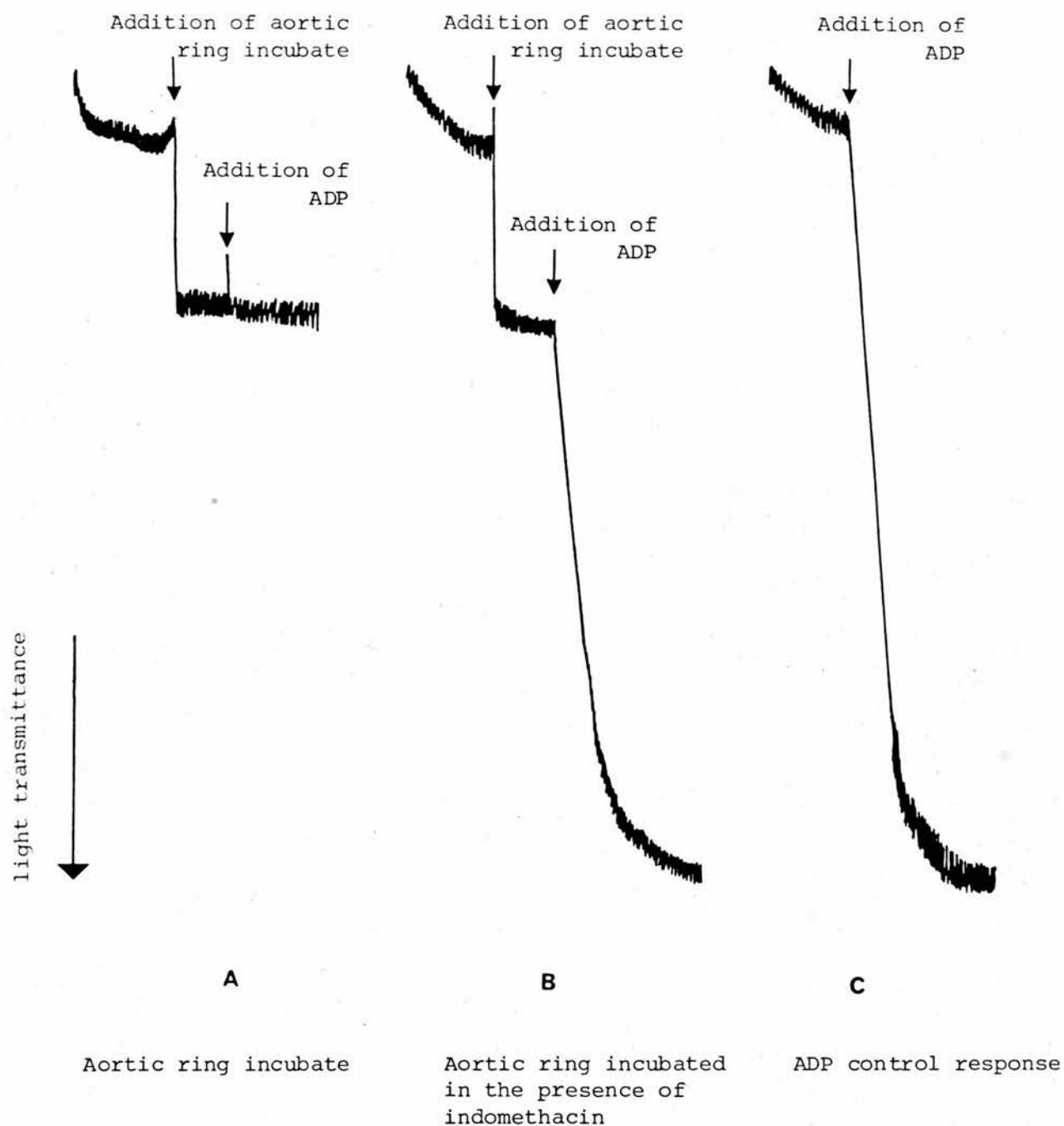


Figure 5.1 Platelet aggregation assay system for PGI_2

gation response induced by ADP. PGI_2 is the most potent anti-aggregatory naturally occurring prostaglandin. It is considerably more potent than PGD_2 or PGE_1 (Moncada, Vane and Whittle, 1977).

It was initially determined that transfer of 0.05 ml of the aortic ring incubate to the platelets, followed by addition of ADP, resulted in a slight inhibitory response whereas transfer of 0.1 ml completely abolished the ADP response. In all subsequent determinations, 0.1 ml of the incubate was transferred - this dose was considered suitable as it would completely inhibit the ADP response but was not a higher dose than was necessary to cause this effect.

Any compound which inhibited either the prostaglandin cyclo-oxygenase or PGI_2 synthetase enzymes should result in an aggregation response on the addition of ADP. This was ascertained using the prostaglandin cyclo-oxygenase inhibitor indomethacin ($20\ \mu\text{M}$) (Figure 5.1). After pre-incubation of the aortic rings with either 8,11,12-THETA, 8,9,12-THETA or EPHETA (at a concentration of $10\ \mu\text{g.ml}^{-1}$ in the aortic incubate), incubation with arachidonic acid and transfer of 0.1 ml of the incubate to the platelet aggregometer, the ADP response was completely abolished. (The 8,9,12-THETA sample was treated with

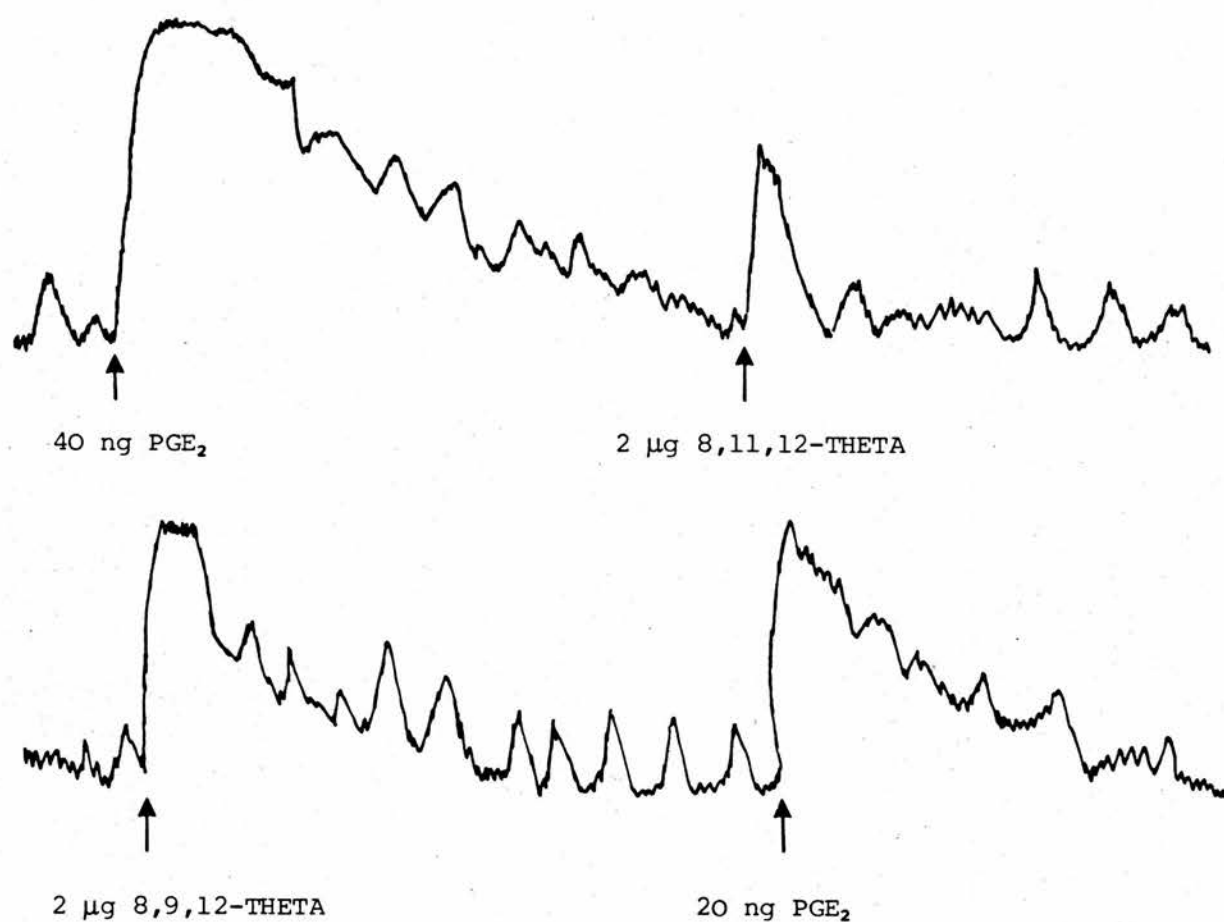


Figure 5.2 Responses of PGE₂, 8,11,12-THETA and 8,9,12-THETA on the rat fundus

base as described in Section 5.2.1 before testing). This indicated that, at this concentration, these compounds had no inhibitory effect on either the prostaglandin cyclo-oxygenase or PGI₂ synthetase enzymes.

3. Tissue Preparations

The tissue preparations examined were the rabbit aorta which contracts in response to thromboxane A₂ and the prostaglandin endoperoxides (Needleman et al, 1976) and the rat fundus and guinea pig ileum which contract in response to the classical prostaglandins PGE₂ and PGF_{2α} (Horton and Main, 1965; Gilmore, Vane and Wyllie, 1968). The sensitivity of the rabbit aorta was ascertained using a 50 ng dose of 11,9-epoxymethano-PGH₂ (a thromboxane A₂ mimic) and the sensitivity of the other two tissues was ascertained using PGE₂ (10-40 ng) and PGF_{2α} (20-40 ng).

At a dose of 1 μg EPHETA and 2 μg Zone III, there were no effects observed on any of the assay tissues. On the rabbit aorta, doses of 1 and 2 μg 8,11,12-THETA and 8,9,12-THETA did not produce any response. The 8,11,12-THETA solution, at doses of 1 and 2 μg 8,11,12-THETA, produced minimal contractions on the rat fundus (less than the response of 20 ng PGE₂ or 40 ng PGF_{2α}). The small amounts of thromboxane B₂

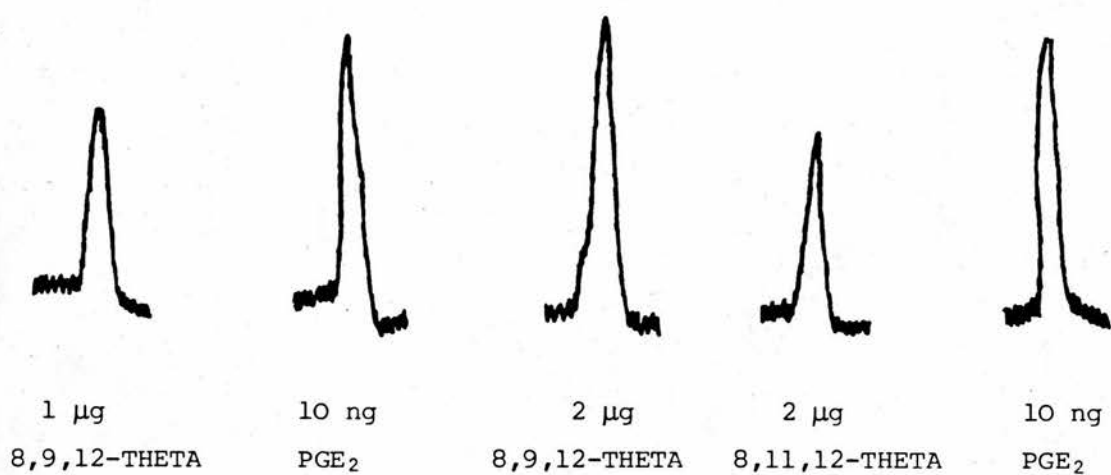


Figure 5.3 Responses of 8,9,12-THETA, PGE_2 and 8,11,12-THETA on the guinea pig ileum

present in the 8,11,12-THETA solution did not account for these contractions (Boot, Dawson, Cockerill, Mallen and Osborne, 1977). On the rat fundus, the 8,9,12-THETA solution at a dose of 1 μ g 8,9,12-THETA produced a minimal response. Doubling the dose produced a response equivalent to 20 ng PGE₂ (Figure 5.2). On the guinea pig ileum, doses equivalent to 1 and 2 μ g 8,11,12-THETA produced minimal contractions (less than the response of 10 ng PGE₂, 40 ng PGF_{2 α}) and 2 μ g 8,9,12-THETA caused a similar contraction to 10 ng PGE₂ (Figure 5.3).

It was initially considered possible that the 8,9,12-THETA solution was contaminated with small amounts of PGE₂. However, treatment of the solution under basic conditions (which destroyed the activity of a PGE₂ standard) did not result in any loss of activity. GC-MS examination of the Me/BuO/TMS derivative of a sample (25 μ g 8,9,12-THETA) of the solution by selected ion monitoring confirmed the absence of PGE₂ in the sample.

In summary, none of the newly identified arachidonic acid metabolites tested had potent contractile activity on either the rabbit aorta, rat fundus or guinea pig ileum.

4. Chemotaxis

Introduction

Chemotaxis can be defined as a reaction by which the direction of locomotion of cells is determined by substances in their environment. In contrast, chemokinesis can be defined as a reaction by which the speed or frequency of locomotion and/or the frequency and magnitude of turns of cells is determined by substances in their environment. Chemokinetic responses can arise from the increase in velocity of cells moving at random, whereas chemotactic responses are caused by a concentration gradient (Wilkinson and Allan, 1978). In inflamed tissues, it is stated that chemotactic substances attract leukocytes from vessels to the site of inflammation.

Perhaps the most common method for measuring chemotaxis in vitro is that devised by Boyden (1962). Although the Boyden chamber has undergone modification from the original design, the principle remains the same. The basic concept of assaying chemotaxis by this method is very simple and involves incubating the cells in the presence of different stimuli and determining and comparing the migration of cells in different samples.

Two principal counting methods for determining chemotactic effects are used. The first method involves counting the number of cells that have reached the lower surface of the filter after a given time period. The second, referred to as the leading front technique (Zigmond, 1974) involves incubating the cells for a shorter period of time, which allows the cells to migrate into the filter but not to traverse its complete depth. A measure is made of the distance migrated by the leading front two cells. The first technique was used in this study as it was considered it would be the simpler to establish.

Rabbit peritoneal PMNL were used as these cells were the most readily obtained. The chemotaxis assay system clearly required a positive control to check that the cells would respond satisfactorily and initially, it was decided to use PGE₁ for this purpose as Kaley and Weiner (1971); Higgs, McCall and Youlten (1975); Walker, Smith and Ford-Hutchison (1976) had all reported that PGE₁ was chemotactic for these cells.

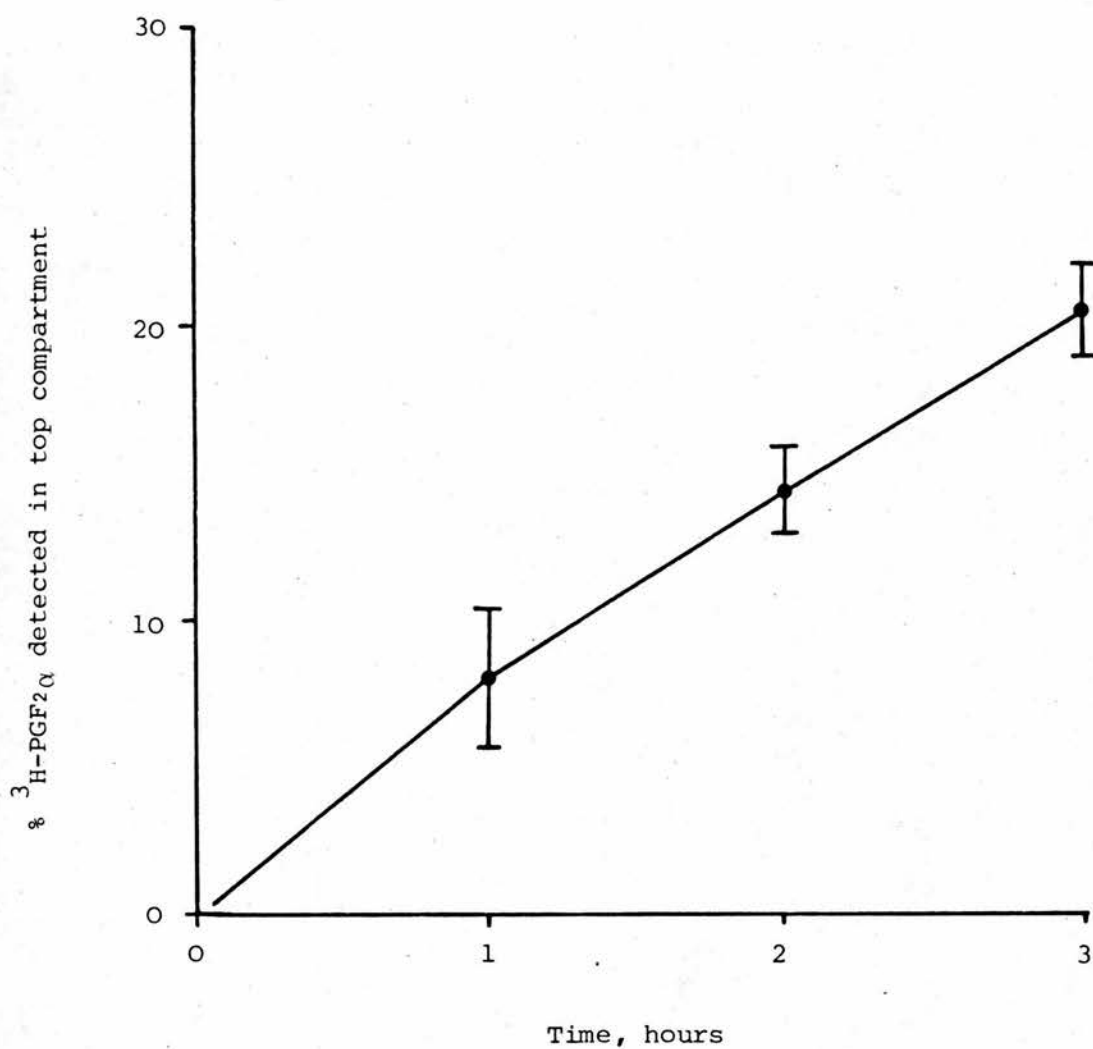
The Search for a Positive Control

In this study, numerous experiments using PGE₁ at concentrations of 1 and 10 $\mu\text{g}.\text{ml}^{-1}$ indicated that this substance was exerting neither a chemotactic

nor chemokinetic influence and was therefore not useful as a positive chemotactic agent. Reports by a group of Japanese workers (Shibuya, Masuda and Izawa, 1976) and Diaz-Perez et al (Diaz-Perez, Goldyne and Winkelmann, 1976) indicated that they had also been unable to demonstrate that PGE_1 was chemotactic for rabbit PMNL.

A similar controversy existed on the chemotactic effect of $\text{PGF}_{2\alpha}$ (Higgs et al, 1975; Shibuya et al, 1976; Diaz-Perez et al, 1976) for rabbit PMNL. In this study, experiments using $\text{PGF}_{2\alpha}$ at doses of 1 and $10 \mu\text{g}.\text{ml}^{-1}$ indicated that there was no chemotactic response for the rabbit cells.

Due to the failure of PGE_1 and $\text{PGF}_{2\alpha}$ to elicit a response, a positive chemotactic agent was still required to check that the cells would respond satisfactorily. Shibuya et al (1976) used casein as their positive control and determined that the optimum dose was 0.2% (w/v) casein. Although this concentration was very high and the substance was not related structurally to the compounds to be tested, the optimum dose of casein by the Japanese workers was found to induce both a chemokinetic and chemotactic response in this study. Casein was therefore used as a positive control in all subsequent experiments. It is noteworthy that the chemo-



Note: the values shown are the mean (\pm standard deviation) from three separate experiments

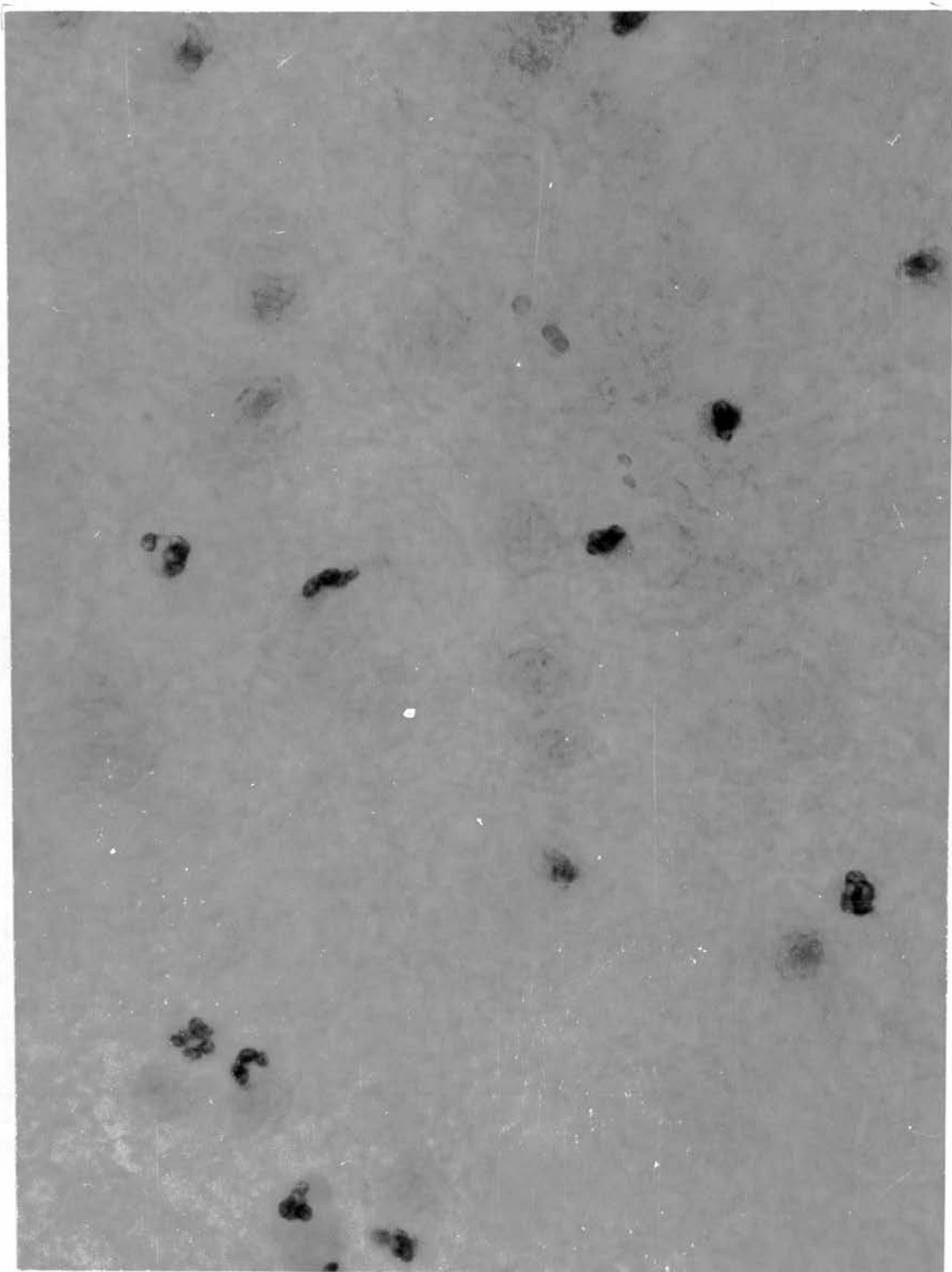
Figure 5.4 Equilibration of PGF $_2\alpha$ in the Boyden chamber

tactic constituents of crude casein have not been identified although it has been observed that the activity was easily extracted into organic solvents. An examination of the extract by gas chromatography indicated that several free fatty acids were present (Turner and Lynn, 1978), one or a number of which may be responsible for the chemotactic activity. The responses of PGE_1 and $\text{PGF}_2\alpha$ were determined on several occasions using casein as a positive control and confirmed the above observations concerning their lack of activity. Despite many reservations about this use of casein, on two occasions during the subsequent studies, casein produced only a very slight response and indicated that the cells were not responding in the normal manner.

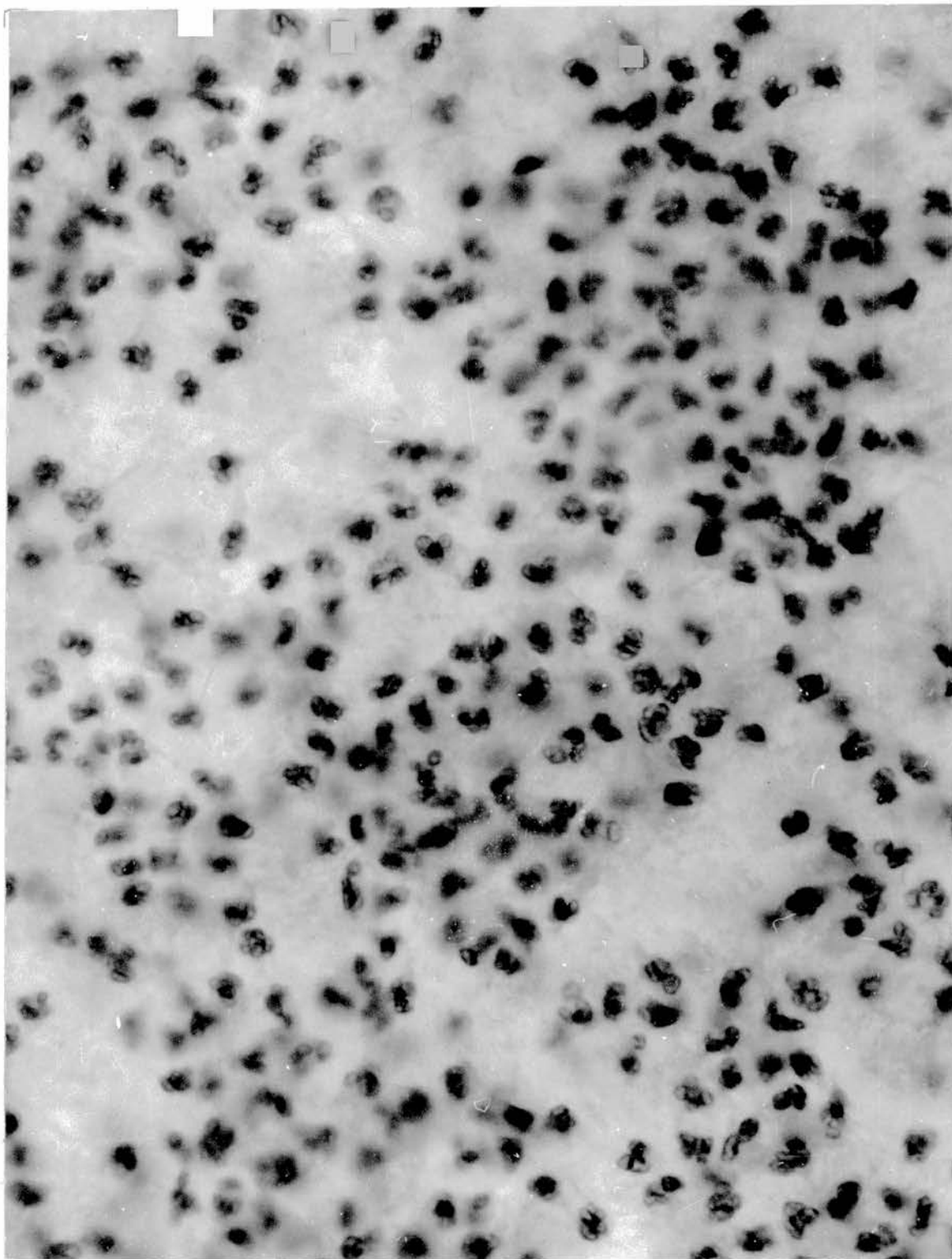
A negative control in which chambers containing only fortified TC199 were prepared, was demonstrated to be necessary as cell counts from this sample varied considerably. Occasionally, the cell counts were very high and the results from these experiments were therefore meaningless. Normally, the background count was only one or two cells per high power field.

Equilibration Studies

A disturbing report (Shibuya et al, 1976) suggested that the concentration gradient of $\text{PGF}_2\alpha$ (as



Control chamber
EPHETA 5 $\mu\text{g}.\text{ml}^{-1}$



Test Chamber
EPHETA $5 \mu\text{g}.\text{ml}^{-1}$

Figure 5.5 Photographs of microscope slide preparations
illustrating the chemotactic effect of EPHETA

TABLE 5.1

Chemotaxis results

Chamber	Experiment 1 cell counts	Experiment 2 cell counts
Fortified TC199 (negative control)	9.8 \pm 1.0	1.8 \pm 0.4
	8.8 \pm 1.4	2.2 \pm 0.5
Casein test (positive control)	\sim 200*	50.4 \pm 3.1
	\sim 200*	
Casein control	47.8 \pm 2.2	36.4 \pm 3.4
	47.0 \pm 7.2	
EPHETA test (5 $\mu\text{g}.\text{ml}^{-1}$)	\sim 200*	33 \pm 6.3
	\sim 200*	
EPHETA control (5 $\mu\text{g}.\text{ml}^{-1}$)	18.2 \pm 1.1	10.8 \pm 0.9
	20.6 \pm 3.4	
EPHETA test (1 $\mu\text{g}.\text{ml}^{-1}$)		14.6 \pm 1.1
		20.0 \pm 2.5
EPHETA control (1 $\mu\text{g}.\text{ml}^{-1}$)		1.8 \pm 0.4
		1.4 \pm 0.3

Results show the mean number of cells in 5 high-power fields (objective x40) \pm the standard error

When duplicate values are shown, these were obtained from two separate chambers

* Too many cells to count accurately

measured by RIA) in the Boyden chamber was not maintained over the 3 hour incubation period. As the concentration gradient is crucial to the concept of chemotaxis, these experiments were repeated in this study using $\text{PGF}_{2\alpha}$ ($1 \mu\text{g}.\text{ml}^{-1}$) containing $^3\text{H}\text{-PGF}_{2\alpha}$ as tracer. Scintillation counting of samples from both compartments of the chamber after different incubation times indicated that less than 20% of the $\text{PGF}_{2\alpha}$ had migrated after 3 hours (Figure 5.4) and that a significant concentration gradient was maintained during the course of the experiments.

The Effect of the Newly Identified Arachidonic Acid Metabolites

Neither 8,11,12-THETA nor 8,9,12-THETA, at a concentration of $1 \mu\text{g}.\text{ml}^{-1}$ showed any increase in cell count over blank values in either the control or test chambers. The EPHETA solution however produced a dramatic visible response at concentrations of 1 and $5 \mu\text{g}.\text{ml}^{-1}$ EPHETA (Figure 5.5). When the cells were counted, control values were seen to be very similar to the blank values (Table 5.1) and it was immediately apparent that a significant chemotactic influence was being exerted. Further purification of the zone of the liquid-gel chromatographic separation containing EPHETA (Zone II, Figure 3.6) was achieved by HPLC. Two isomers of EPHETA were resolved during

this chromatographic purification (Section 4.2). Chemotactic testing of the fractions containing each of two isomers indicated that virtually all activity was attributable to the fractions containing the more polar HPLC isomer. The HPLC purification procedure had removed any traces of 12-HETE from the two epoxide isomers. This was significant as reports (Turner et al, 1975; Turner and Lynn, 1978; Goetzl, Woods and Gorman, 1977; Goetzl and Gorman, 1978) indicated that 12-HETE was chemotactic for human peripheral blood PMNL. The presence of activity in the HPLC purified material provided very strong evidence that the observed activity was indeed due to EPHETA.

It was possible that the 12-HETE prepared by Turner et al (from incubating washed platelets with arachidonic acid) may have contained EPHETA as only one TLC purification was employed. Therefore, their purification method was repeated in this study and indicated that their 12-HETE should have been satisfactorily resolved from any EPHETA that might have been present in their original extract.

Concluding Remarks

It is considered worth emphasising that the Boyden chamber can only satisfactorily be used with all the controls that are employed in this study. Several

workers are guilty of not employing all these controls, which has resulted in confusion in the interpretation of data. Care must be taken in preparing fresh solutions of compounds for testing as, for example, "aged PGE₁ solutions" have been reported to show increased chemotactic activity (Walker et al, 1976). Lack of these precautions may in part explain some of the conflicting results.

In view of the many conflicting results obtained using the Boyden chamber (in addition to those previously mentioned), the reader is referred to Kitchen, Boot and Dawson, 1978; Goetzl and Gorman, 1978 concerning thromboxane B₂), it is intended that further work will be carried out on EPHETA to substantiate the effects observed in the Boyden chamber by another method. Chemotactic activity was reported to be accompanied by a concomitant release of the enzymes β -galactosidase and β -glucuronidase (Wilkinson, 1974) and it is hoped to monitor the release of these enzymes during the incubation process.

5. Blood Pressure Experiments

It was of interest to determine whether or not either of the trihydroxy acids or EPHETA had any effect on blood pressure. This interest was stimulated by reports by Wennmalm (1977) and Bayer and Förster (1979) that the vasodepressor effect they obtained from arachidonic acid could not be completely abolished by pre-treatment with indomethacin. It was suggested that an arachidonic acid metabolite which was not a product of the prostaglandin cyclooxygenase pathway might be responsible for these effects.

Blood pressure experiments were carried out on two male cats. The sensitivities of the preparations were checked using PGE_2 ($1 \mu\text{g}$) which caused a fall in blood pressure. Infusion of arachidonic acid ($300 \mu\text{g} \cdot \text{kg}^{-1}$) resulted in an increase in blood pressure. In contrast to the previously mentioned reports, after infusion of indomethacin ($1 \text{ mg} \cdot \text{kg}^{-1}$), the effect of arachidonic acid was completely abolished. After further treatment with indomethacin ($0.5 \text{ mg} \cdot \text{kg}^{-1}$), the dose of arachidonic acid was doubled but the response was still completely blocked by the indomethacin. The increase in blood pressure following arachidonic acid infusion was equivalent to a $5 \mu\text{g}$ dose of $\text{PGF}_{2\alpha}$ and may be attri-

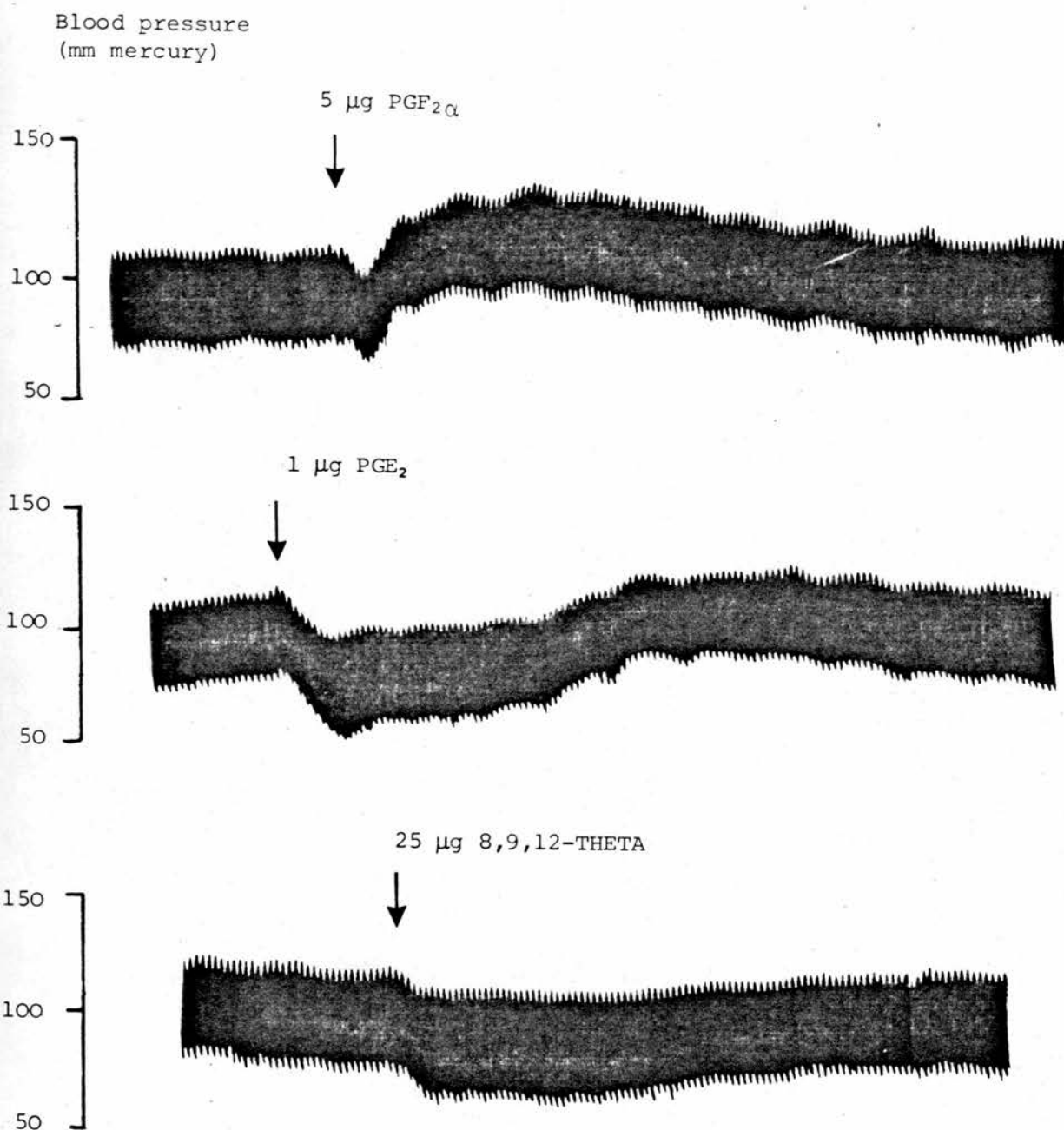


Figure 5.6 The effects of $\text{PGF}_2\alpha$, PGE_2 and 8,9,12-THETA on cat blood pressure

butable to the formation of this prostaglandin, PGD_2 , the prostaglandin endoperoxides or thromboxane A_2 . It is noteworthy that 11,9-epoxymethanoprostaglandin PGH_2 (a thromboxane A_2 mimic) causes an increase in blood pressure in the cat (R. L. Jones, unpublished results).

Infusion of 25 μg of 8,11,12-THETA and EPHETA produced no effect. The 8,9,12-THETA solution, at a dose of 25 μg 8,9,12-THETA, caused a slight decrease in blood pressure (Figure 5.6). This response was slightly less than the effect caused by 0.5 μg PGE_2 . As described in Section 5.2.3., an examination of the 8,9,12-THETA solution by GC-MS indicated that this solution was not contaminated with any significant amounts of PGE_2 and therefore it was concluded that 8,9,12-THETA probably possessed very slight vasodepressor properties. Further purification and re-testing would be necessary for confirmation of this slight activity.

Blood was collected from one of the cats and PRP and washed platelets were prepared as described previously (Section 2.2) and incubated with arachidonic acid. GC-MS examination of the Me/TMS derivative of the products indicated that the platelets formed 12-HETE, EPHETA and THETA. No thromboxane B_2 or HHT were detected which indicated the effectiveness of the indomethacin treatment.

The fairly wide range of biological tests applied to the newly discovered arachidonic acid metabolites resulted only in a pronounced response by EPHETA in the chemotaxis assay.

The small contractile response of the strips of guinea pig ileum and rat fundus to the trihydroxy acid solutions are comparable to the effects of two analogous C18 trihydroxy acids reported by a Russian group of workers (Panosyan, Avetisyan, Mnatzakanyan, Asatryan, Vartanyan, Boroyan and Batrakov, 1979). They isolated their material (a mixture of 9,12,13-trihydroxy-10,15-octadecadienoic acid and 12,15,16-trihydroxy-9,13-octadecadienoic acid) from the roots of a wild plant of the marrow family (Bryonia Alba L). This plant had been used from ancient times in folk medicine and homoeopathy and these workers were attempting to determine the active principles.

A difficult problem in this area of pharmacological research is to decide which biological tests to apply. There is always the possibility that one or more of these compounds may possess activity in a biological system which was not investigated in this study. An obvious limitation is the amount of material necessary for thorough biological screening. The large scale incubations employed in this study were only adequate for the

preparation of microgram quantities of the novel metabolites. It is an additional problem, when biological activity is detected in samples from biological extracts, to prove that the activity is indeed due to the metabolites under test. When biological activity is detected, it is necessary to determine the effect(s) on the observed activity of further chromatographic purification(s) of the sample.

There is also the possibility, in analogy with the products resulting from the prostaglandin cyclo-oxygenase pathway, that an intermediate compound (such as the THETA intermediate) may possess considerably more biological activity than the stable end products. This type of investigation is clearly a possible area for further work.

SECTION 6

CONCLUDING REMARKS

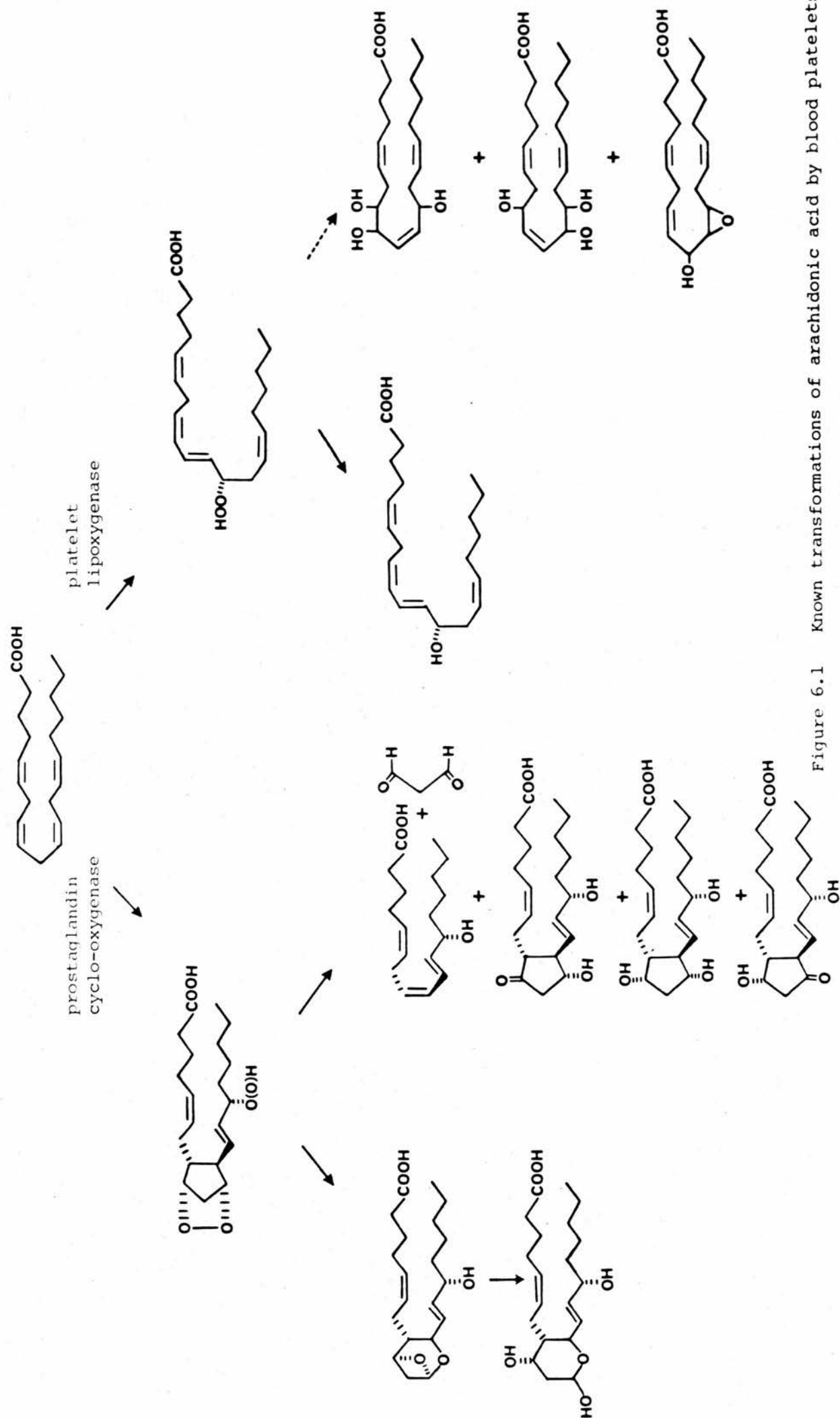


Figure 6.1 Known transformations of arachidonic acid by blood platelets

Two trihydroxy acids (8,11,12-THETA and 8,9,12-THETA) and an epoxy-hydroxy acid (EPHETA) have been detected and identified as novel metabolites of arachidonic acid from incubations with washed blood platelets from human, horse, dog, cat and rabbit. It was demonstrated that these products did not arise from the prostaglandin cyclo-oxygenase pathway as their formation was not inhibited by indomethacin and the most likely method of formation involved further transformation of 12-HPETE.

The known transformations of arachidonic acid by blood platelets are summarised in Figure 6.1. Enzymes have been characterised for the conversion of arachidonic acid to the prostaglandin endoperoxides, conversion of the endoperoxides to thromboxane A₂ and HHT, and conversion of arachidonic acid to 12-HPETE (Hammarström and Falardeau, 1977; Nugteren, 1975). The transformation of 12-HPETE to 12-HETE was considered by Nugteren to be both enzymic and non-enzymic. Further work requires to be carried out to determine whether or not the other products are formed enzymatically.

It is of interest to note that Falardeau et al (1976) tentatively identified the corresponding trihydroxydienoic acids (THEDA) to THETA from incubations of dihomo- γ -linolenic acid with washed human platelets but did not establish a similar conversion from arachidonic acid. They also identified two dihydroxy-trienoic acids as products but did not detect a compound analogous to EPHETA.

The analogous dihydroxy acids derived from arachidonic acid have not been detected in this study. While dihomo- γ -linolenic acid is a good substrate for the platelet lipoxygenase (Nugteren, 1975), it is a much poorer substrate for the prostaglandin cyclo-oxygenase than arachidonic acid (Falardeau et al, 1976). As a result, under similar incubation conditions, relatively more dihomo- γ -linolenic acid (in comparison to arachidonic acid) would be available for the platelet lipoxygenase and therefore THEDA formation may be more pronounced than THETA formation, which may explain why these workers only detected the dihomo- γ -linolenic acid product.

The role(s) of THETA and EPHETA in platelet functions like the role(s) of 12-HPETE and 12-HETE are not understood. It is interesting that both the platelet epoxide EPHETA and 12-HETE have been found to possess chemotactic activity for rabbit peritoneal and human peripheral blood PMNL respectively. It has still to be determined if EPHETA is chemotactic for human peripheral blood PMNL and also if 12-HETE is chemotactic for rabbit peritoneal PMNL. The role(s) of hydroxylated and epoxidised fatty acids as chemoattractants and the determination of which compounds possess this type of activity are relatively unexplored subjects.

The recent investigations of workers at the Karolinska Institute concerning the metabolism of arachidonic acid by rabbit peritoneal and human peripheral blood PMNL (Borgeat

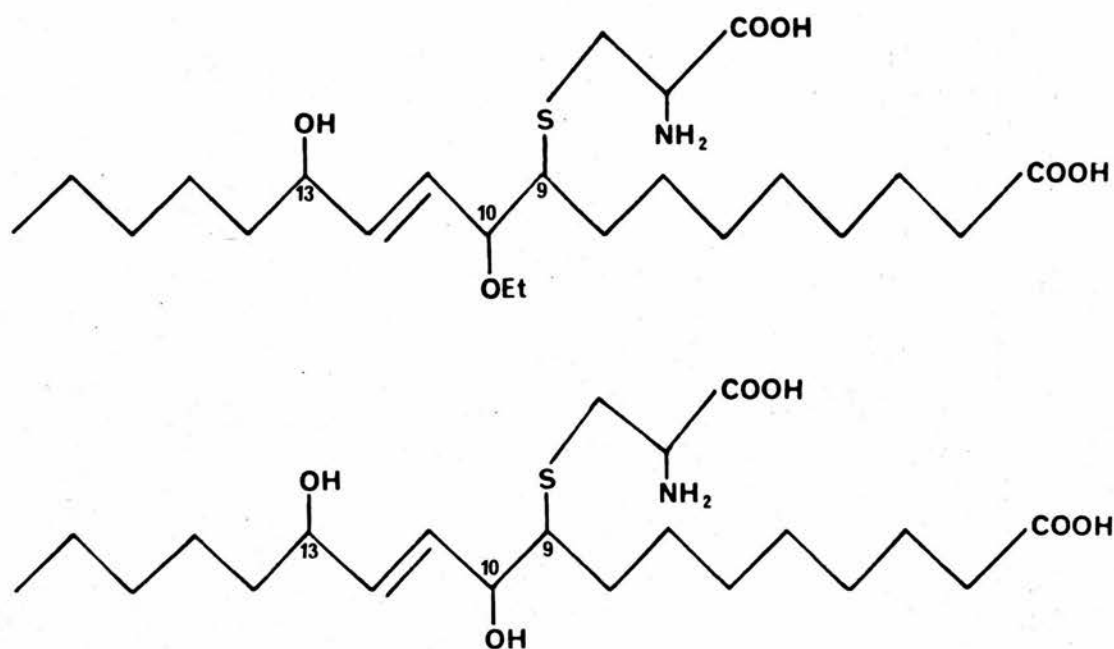


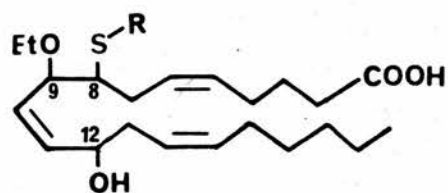
Figure 6.2a Cysteinyl adducts of linoleic acid hydroperoxide



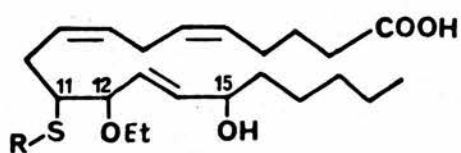
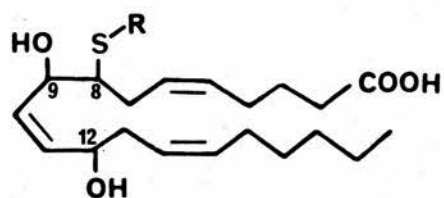
Figure 6.2b Additional product identified (following methylation)

and Samuelsson, 1976, 1979, 1979a and 1979b). have resulted in the identification of a number of hydroxylated and epoxidised arachidonic acid derivatives as products: 5-HETE, 5,12-DHETE, 15-HETE and a 5,6-epoxy compound (Leukotriene A). It was proposed (Samuelsson, Borgeat, Hammarström and Murphy, 1979) that the unstable 5,6-epoxy compound (Leukotriene A) was formed from 5-HPETE and was the precursor to SRS (Leukotriene C). Leukotriene C was demonstrated to be a novel cysteinyl derivative of arachidonic acid with a hydroxyl group at C-5, three conjugated and one isolated double bonds with the amino acid linked as a thioether to C-6 (Figure 1.4). Further work will doubtless be centred on this newly identified pathway - it is noteworthy that it still has to be demonstrated that Leukotriene C and SRS-A are identical.

It should be possible to chemically prepare cysteinyl adducts of arachidonic acid (other than Leukotriene C) including the adduct of the platelet epoxide EPHETA. It will then be interesting to compare the biological activity of a range of cysteinyl adducts with Leukotriene C.



12-HPETE
products



15-HPETE
products

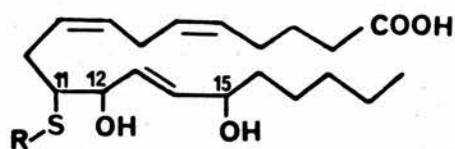


Figure 6.3 Analogous reaction products of 12-HPETE and 15-HPETE

It is interesting to note that the formation of cysteinyl adducts from linoleic acid hydroperoxide (13-hydroperoxide) following treatment with cysteine in the presence of ferric chloride in 20% aqueous ethanol under nitrogen has been reported (Figure 6.2a). The corresponding N-acetyl cysteinyl adducts were isolated when N-acetyl cysteine was used in place of cysteine (Gardner, Weisleder and Kleiman, 1976; Gardner, Kleiman, Weisleder and Inglett, 1977). When the reaction with cysteine included both isomers of linoleic acid hydroperoxide (13- and 9-hydroperoxide) and air, an additional product was isolated (Figure 6.2b) (Gardner et al, 1977). The formation of these adducts is not analogous to the previously mentioned leukotriene pathway. However, this reaction is a potential route for the preparation of other cysteinyl derivatives of arachidonic acid. The analogous reaction products of 15-HPETE and 12-HPETE are shown in Figure 6.3. It is noteworthy that the 12-HPETE adduct is substituted at the same positions in the carbon chain as 8,9,12-THETA. The preparation of these adducts and the determination of their biological activity is one of many possible areas for further research.

Future interest will certainly be focussed on SRS-A and the newly identified leukotriene pathway, in addition to further work on the platelet lipxygenase and the prostaglandin/thromboxane pathways. It is striking that the vast quantity of information concerning the metabolism

(and metabolites) of arachidonic acid (and dihomog-
linolenic acid) has been acquired over a relatively short
period of time. It is now only 15 years since Van Dorp
et al (1964) and Bergström et al (1964) discovered that
arachidonic acid and dihomog-linolenic acid were
prostaglandin precursors!

The importance of arachidonic acid (and to a lesser extent
dihomog-linolenic acid) metabolites in physiology and
potentially medicine place them in the forefront of
chemical, biochemical and pharmacological investigations.
Despite the rapid advances made during the last 15 years,
the understanding of the physiological roles of these
compounds is far from complete.

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THE IDENTIFICATION OF TRIHYDROXYEICOSATRIENOIC ACIDS AS PRODUCTS FROM THE INCUBATION OF ARACHIDONIC ACID WITH WASHED BLOOD PLATELETS

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ABSTRACT

Arachidonic acid is converted by washed platelets from man, horse and dog into a mixture of 8,9,12-trihydroxy-eicosa-5,10,14-trienoic acid and 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid (termed 8,9,12-THETA and 8,11,12-THETA respectively and THETA collectively). Gas chromatographic - mass spectrometric evidence of structure is discussed.

INTRODUCTION

Rapid metabolism of arachidonic acid occurs in human platelets by two pathways. Fatty acid cyclo-oxygenase generates PGG_2 and PGH_2 which are then enzymatically isomerised to thromboxane A_2 , a very potent stimulant of platelet aggregation (1). Lipoyxygenase action also produces 12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid which is subsequently transformed to 12-hydroxyeicosa-5,8,10,14-tetraenoic acid (HETE) (2). Our studies with human, horse and dog platelets have revealed the presence of further novel metabolites of arachidonic acid.

EXPERIMENTAL PROCEDURE AND RESULTS

Platelet rich plasma and washed platelets from citrated human, horse and dog blood were prepared according to Hamberg *et al* (3). Washed platelets (at a concentration of 5×10^8 - $5 \times 10^9 \text{ ml}^{-1}$) were incubated with arachidonic acid ($1.65 \times 10^{-4} \text{ M}$) at 37°C for 30 min. In some experiments $1\text{-}^{14}\text{C}$ -arachidonic acid (final sp. activity $120 \mu\text{Ci mmole}^{-1}$) was added as tracer to incubates.

PROSTAGLANDINS

After acidification, the products were extracted with diethyl ether; non-polar lipids were then removed by partition between 67% ethanol and petroleum ether.

Initial work, which involved purification of the methyl ester of the incubation extract by liquid-gel partition chromatography (4) and HPLC (Partisil PAC, hexane/isopropanol 4:1), afforded a single zone of radioactivity, slightly less polar than $\text{PGF}_{2\alpha}$, which was free of PGD_2 , PGE_2 , $\text{PGF}_{2\alpha}$ and TXB_2 . GC-MS studies indicated the presence of a mixture of 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid (8,9,12-THETA) and 8,11,12-trihydroxy-eicosa-5,9,14-trienoic acid (8,11,12-THETA). Complete separation of the two positional isomers was subsequently achieved by liquid-gel chromatography of the free acid of the incubation extract. The data discussed below refer to the separated positional isomers.

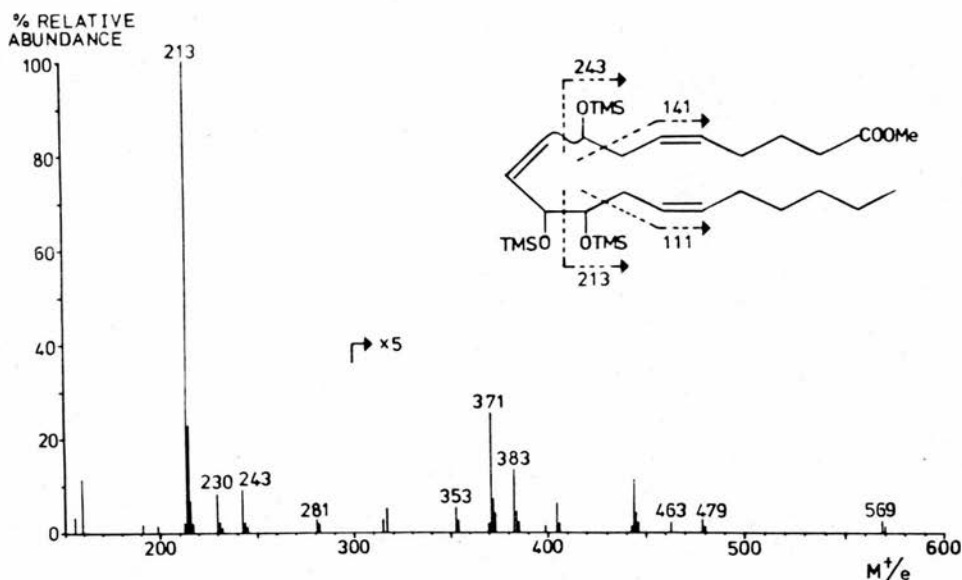


Fig. 1. Mass spectrum of 8,11,12-THETA (Me/TMS).

Gas chromatography of the methyl ester-trimethylsilyl ether (Me/TMS) derivatives of both compounds showed two partially resolved peaks (carbon values 23.6 and 23.8 on a

3% OV1 column). From mass spectrometric analysis the partially resolved GC peaks were shown to be isomers with very similar mass spectra. The Me/TMS derivative of the more polar positional isomer on liquid-gel chromatography showed prominent ions in both mass spectra taken at carbon values 23.6 and 23.8 at m/e 569 (M-15), 479 (M-15-90), 463 (M-31-90), 383 (M-90-111), 371 (M-213), 353 (M-90-141), 281 (M-90-213), 243 (CH(OTMS) CH₂ CH = CH (CH₂)₃ COOMe), 230 (M-141-213 or -111-243), 213 (CH(OTMS) CH₂ CH = CH C₅H₁₁) (base peak). (Fig. 1). These mass spectra are consistent with isomers of 8,11,12-THETA. The ethyl ester-trimethylsilyl ether spectra were consistent with these assignments.

The Me/TMS derivative of the less polar positional isomer on liquid-gel chromatography showed prominent ions in both mass spectra taken at carbon values 23.6 and 23.8 at m/e 569 (M-15), 479 (M-15-90), 463 (M-31-90), 383 (M-90-111), 353 (M-90-141), 251 (M-90-243), 243 (CH(OTMS) CH₂ CH = CH (CH₂)₃ COOMe) (base peak) 230 (M-141-213 or -111-243), 213 (CH(OTMS) CH₂ CH = CH C₅H₁₁) (Fig. 2).

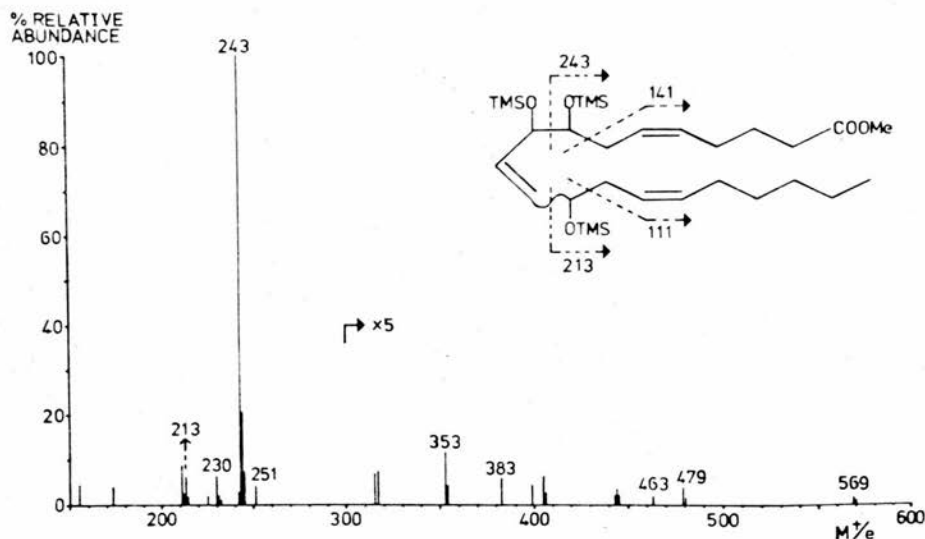


Fig. 2. Mass spectrum of 8,9,12-THETA (Me/TMS).

PROSTAGLANDINS

These mass spectra are consistent with isomers of 8,9,12-THETA. The ethyl ester-trimethylsilyl ether spectra were consistent with these assignments. It should be noted that the Me/TMS derivative of 9,10-dihydroxystearic acid undergoes one major fragmentation in the mass spectrometer - between C9 and C10, indicating that fragmentation takes place preferentially between two adjacent TMS-substituted hydroxyl groups on the chain of a fatty acid.

Following hydrogenation (platinum oxide/methanol) and Me/TMS derivitisation both compounds still showed two partially resolved GC peaks (carbon values 24.4 and 24.7). The mass spectra indicated that the partially resolved GC peaks were isomers and the spectra of both compounds indicated the presence of three double bonds in the original materials. The mass spectra of the 8,11,12-THETA isomers showed a base peak at m/e 285 (M-215-90), and large ions at m/e 215 (CH(OTMS) C_8H_{17}) and 245 (CH(OTMS) $(CH_2)_6$ COOMe). In the spectra of the 8,9,12-THETA isomers m/e 255 (M-245-90) was the base peak and large ions were again observed at m/e 215 and 245. Multiple ion detection of the 245 and 215 ions of both compounds (Fig. 3) supported the existence of isomers which are suggested to be hydroxy diastereoisomers.

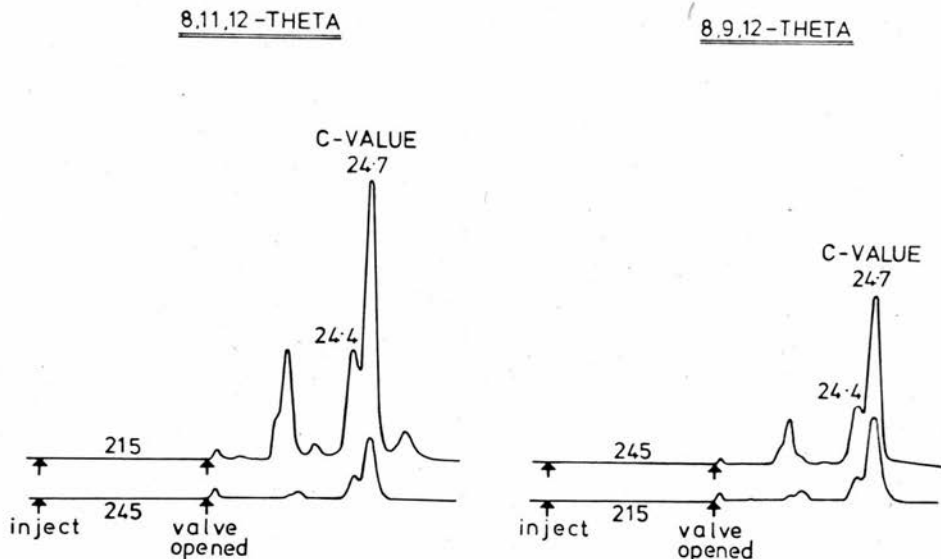


Fig. 3. Multiple ion detection of m/e 215 and 245 ions from 8,11,12-THETA and 8,9,12-THETA (Me/hydrogenated/TMS).

The hydrogenated forms of both compounds formed a *n*-butyl boronate/mono-TMS derivative supporting the presence of vicinal hydroxyl groups. Two partially resolved GC peaks were observed for both compounds (carbon values 26.0 and 26.2). The mass spectra of both GC peaks of this derivative showed m/e 245 as the base peak of 8,11,12-THETA and m/e 215 as the base peak of 8,9,12-THETA. (Fig. 4). Multiple ion detection of the 215 and 245 ions again supported the existence of the stereoisomers as two partially resolved GC peaks were observed for each ion.

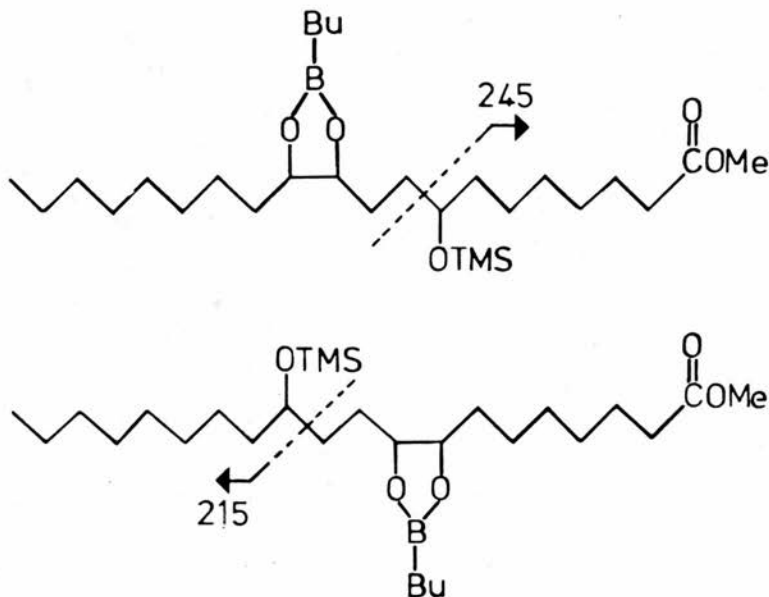


Fig. 4. Fragmentation of 8,11,12-THETA and 8,9,12-THETA (Me/hydrogenated/*n*-butyl boronate/TMS).

The mixture of positional isomers did not react with *O*-methylhydroxylamine, and showed no diene absorption in the ultraviolet region. Further work is required to establish the configurations of the hydroxyl groups and the double bonds (it is probable that the 5,6 and 14,15 bonds have remained cis).

PROSTAGLANDINS

In incubations with washed human platelets THETA was found to be a major metabolite since it has been detected in comparable amounts to HETE and in greater amounts than thromboxane B_2 . Washed horse platelets consistently produced more than 100 times the amount of THETA compared to thromboxane B_2 . Similar material has also been found following incubations of arachidonic acid with sheep uteri and sheep spleen homogenates. The formation of THETA was not blocked by indomethacin ($10^{-5}M$).

It is of interest to note that Samuelsson and co-workers isolated the corresponding trihydroxy-dienoic acids from incubation of bis-homo- γ -linolenic acid with human platelets but were unable to establish a similar conversion from arachidonic acid (5).

A possible mechanism suggested for the formation of THETA is the transformation of 12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid via an epoxy-hydroxy intermediate; analogous conversions of linoleic acid incubated with plant lipoxygenase have been described (6).

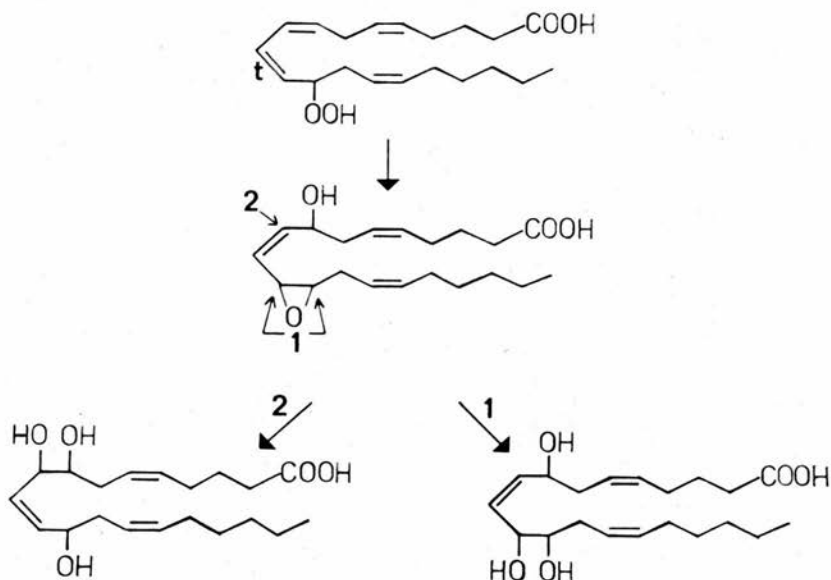


Fig. 5. A possible mechanism of THETA formation.

The biological properties of THETA are under investigation.

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ACKNOWLEDGEMENTS

These studies were supported from an M.R.C. programme grant to Professor E.W. Horton. The technical assistance of Mr. R. Goodall is acknowledged.

NOVEL METABOLITES OF ARACHIDONIC ACID

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ABSTRACT

Arachidonic acid is converted by platelets into a mixture of 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid and 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid (termed 8,11,12-THETA and 8,9,12-THETA respectively and THETA collectively). GC-MS evidence of structure is discussed along with possible precursors of THETA formation and related substances.

INTRODUCTION

Arachidonic acid is the essential fatty acid precursor to the 2-series prostaglandins (1). An alternative metabolic pathway, via a lipoxigenase enzyme, is known to exist in blood platelets of mammals, yielding 12-hydroperoxy- and hence 12-hydroxy-eicosa-5,8,10,14-tetraenoic acid (2). Further study has revealed additional lipoxigenase activity in the conversion of arachidonic acid into 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid and 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid (8,11,12-THETA and 8,9,12-THETA).

METHODS

Platelet rich plasma and washed platelets from citrated human, horse and dog blood were prepared in the usual manner (3). Incubations with radiolabelled 1-¹⁴C arachidonic acid (1.65×10^{-4} M; Sp. Ac. 120 μ Ci/mmol) were performed at 37°C for 30 minutes. After acidification to pH 4, the products were extracted with ether and the ether evaporated. The non-polar lipids were removed by partition of the material between 67% ethanol and petroleum ether. The polar lipids were isolated by evaporation of the alcoholic phase. The material was treated with diazomethane to form the methyl esters and purification by liquid-gel partition chromatography (4) and HPLC gave a single zone of radioactivity slightly less polar than PGF₂ α . The wide unsymmetrical peak was due presumably to the subsequently discovered isomers. This material (THETA) had no diene absorption in the U.V. Gas chromatography-mass spectrometry of the Me₃TMS derivative showed the presence of at least two substances which could be assigned the trihydroxyeicosatrienoic acid constitution. The material formed no oxime derivative. Complete resolution of these isomers was achieved by liquid-gel partition chromatography of the free acid THETA (Fig. 1.).

RESULTS AND DISCUSSION

GC-MS of the separated isomers was performed on 3% OVI. The more polar, major isomer, 8,11,12-THETA, consisted of two partially resolved peaks (C.V. 23.6, 23.8), run as Me,TMS. Both G.C. isomers in this fraction had almost identical mass spectra.

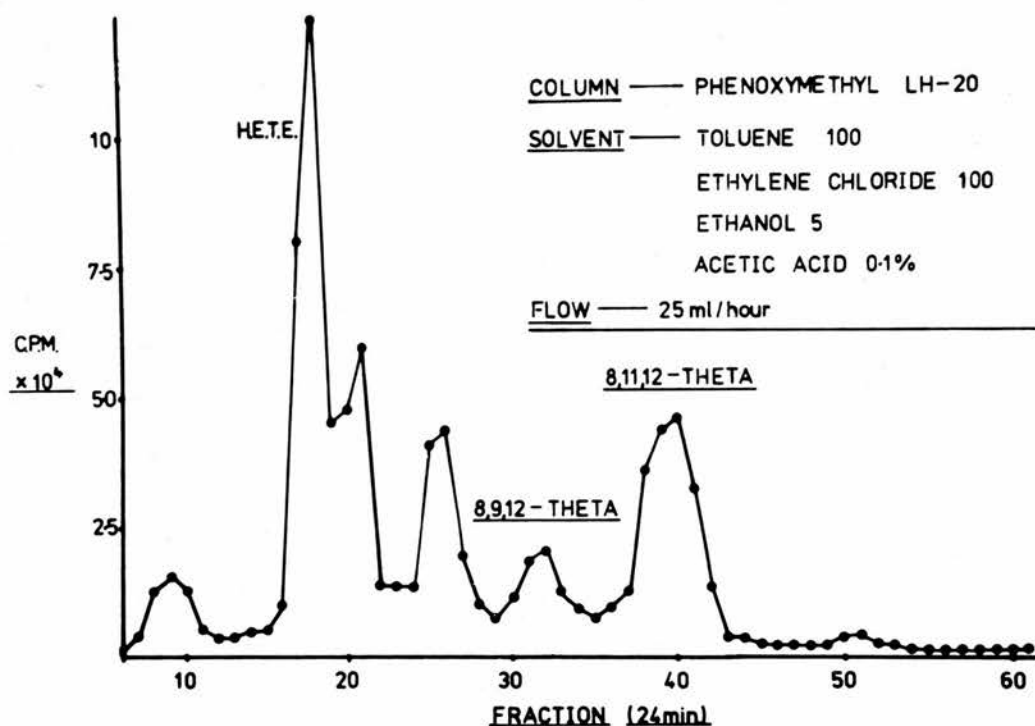


Fig. 1. Chromatogram of polar lipid fraction run as free acids

The structural analysis is shown in Fig.2., the large peak at m/e 213 being the well documented favourable cleavage between the vicinal-OTMS groups. The less polar, minor isomer, 8,9,12-THETA, showed very similar G.C. profile, again with two partially resolved peaks (C.V. 23.6, 23.8) with almost identical mass spectra (Fig. 3).

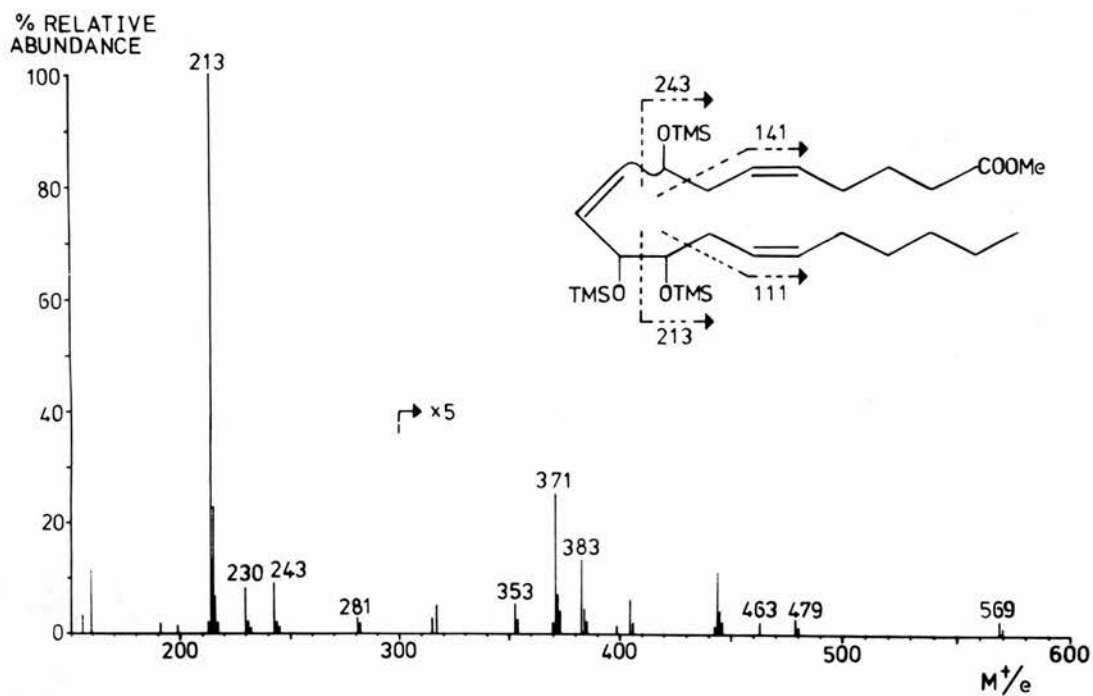


Fig. 2. M.S. of 8,11,12-THETA Me,TMS

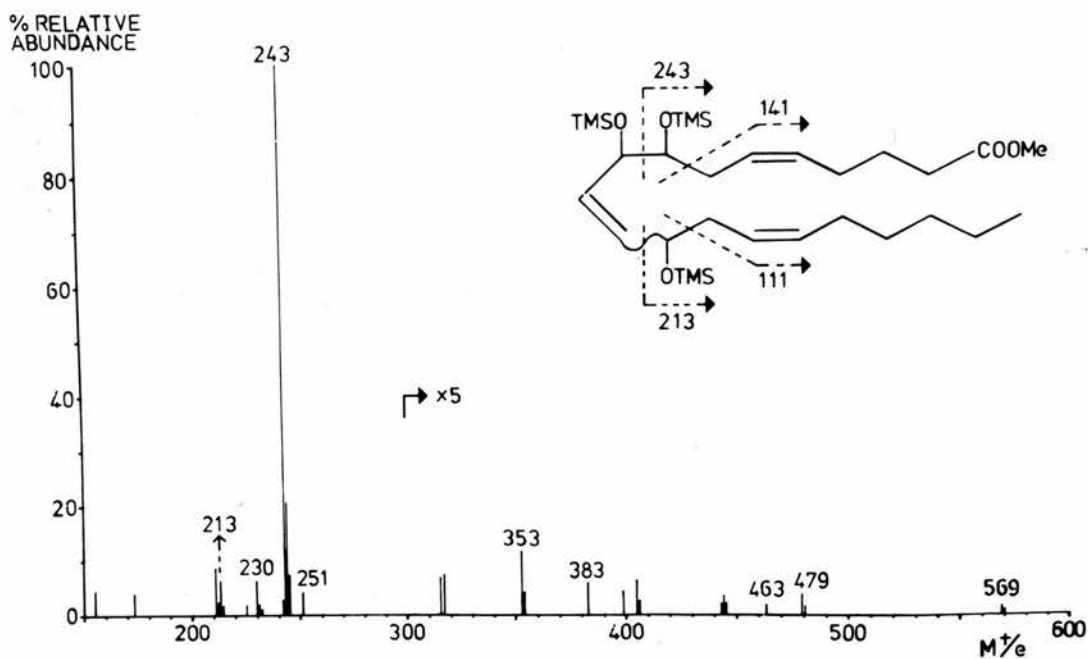


Fig. 3. M.S. of 8,9,12-THETA Me,TMS

The 8,9,12-THETA isomers are characterised by the large peak at m/e 243. All peak assignments in all isomers were checked using the Et,TMS derivatives.

The remaining isomerism in the substances is thought to be due to hydroxyl diastereoisomers. However, it was necessary to remove the possibility of cis-trans isomerism by hydrogenation of all olefinic bonds. Both THETA molecules took up 3 molecules of hydrogen in the presence of platinum but still produced two peaks on G.C. (Fig. 4).

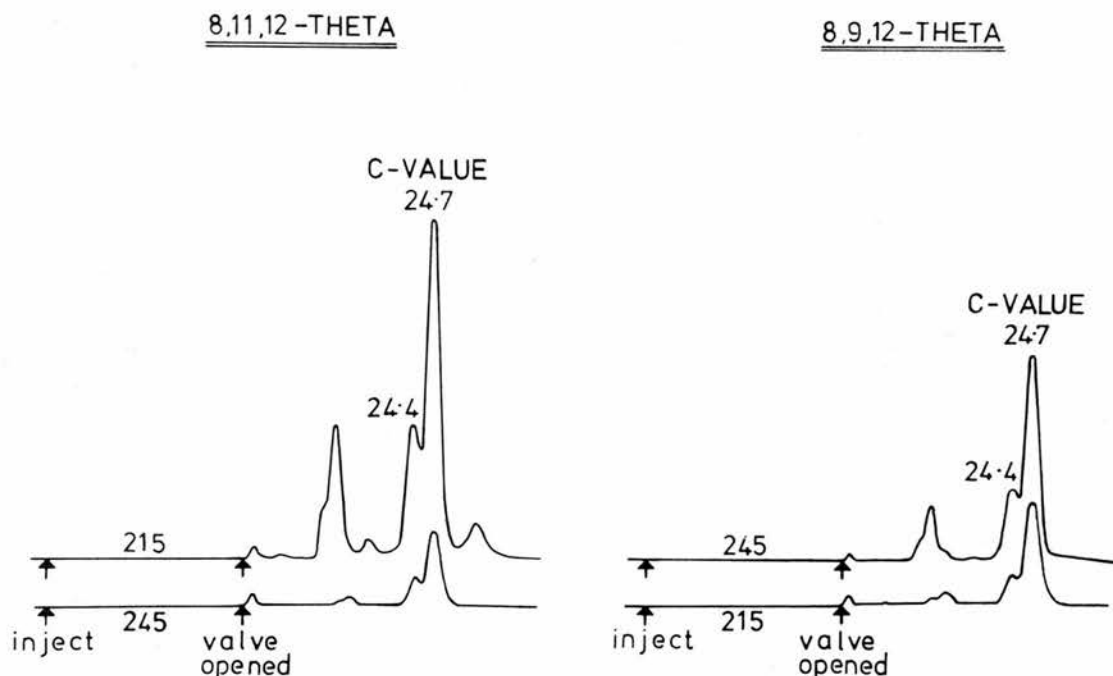


Fig. 4. M.I.D. of m/e 215 and 245 ions from hydrogenated 8,11,12-THETA and 8,9,12-THETA run as Me,TMS derivatives

The mass spectra of the hydrogenated THETA as Me,TMS and the structural analysis is given in Figs. 5 and 6. In the 8,11,12-isomers the base peak m/e 285 is due to (M-215-90), the peak at m/e 267 is (M-143-90 x 2). All other fragmentations are fairly simple cleavages due to loss of 90 a.m.u. (TMSOH) and 31 a.m.u. (OMe). Similarly in the 8,9,12-isomers the base peak m/e 255 is due to (M-245-90) and the peak at m/e 387, (M-113-90). These assignments were checked with the Et,TMS derivatives.

This GC-MS evidence leaves little doubt that the isomerism remaining in the positional isomers of THETA is due to diastereoisomerism at the hydroxyl groups.

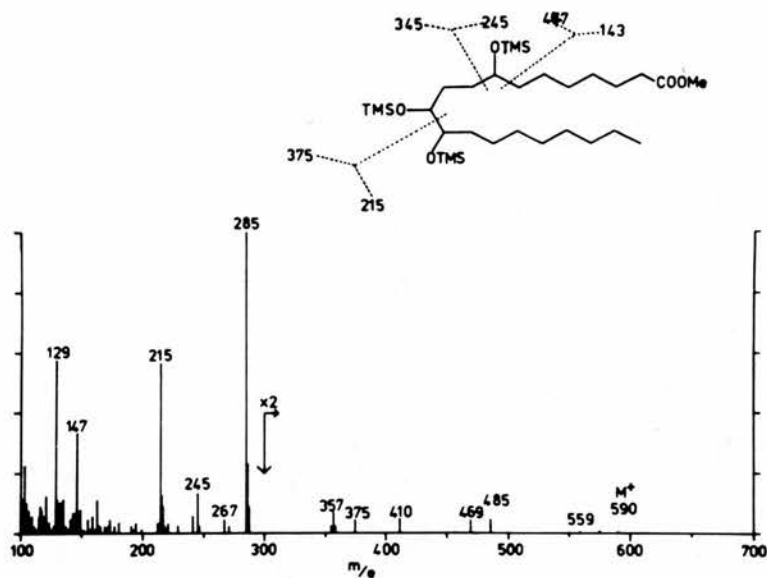


Fig. 5. M.S. of hydrogenated 8,11,12-THETA as Me,TMS (C.V. = 24.4, 24.7)

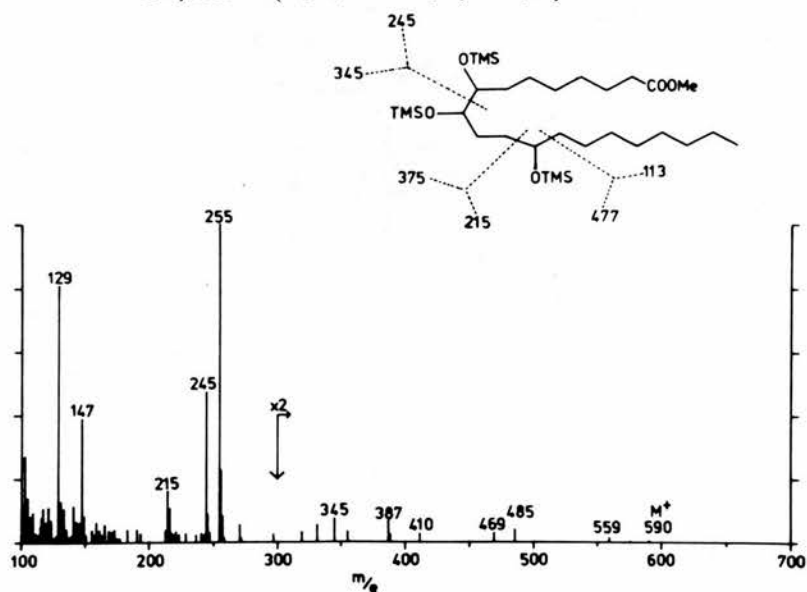


Fig. 6. M.S. of hydrogenated 8,9,12-THETA as Me,TMS (C.V. = 24.4, 24.7)

Final structural proof of the hydroxyl positions was provided by altering the mass spectral fragmentation of the hydrogenated THETA by formation of the cyclic *n*-butyl boronate ester. This is known only to form with 1,2 or 1,3-diols. The mass spectra and the analysis are given in Figs. 7 and 8.

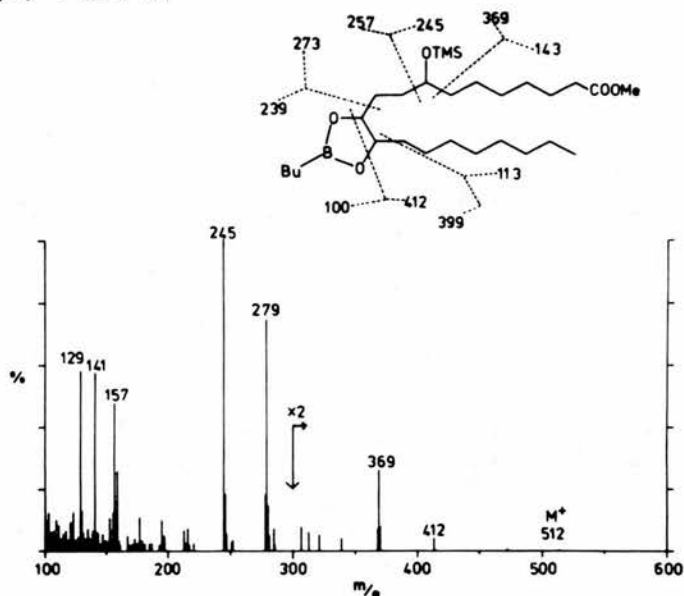


Fig. 7. M.S. of hydrogenated 8,11,12-THETA as Me,nBB,TMS derivative (C.V. = 26.0, 26.2)

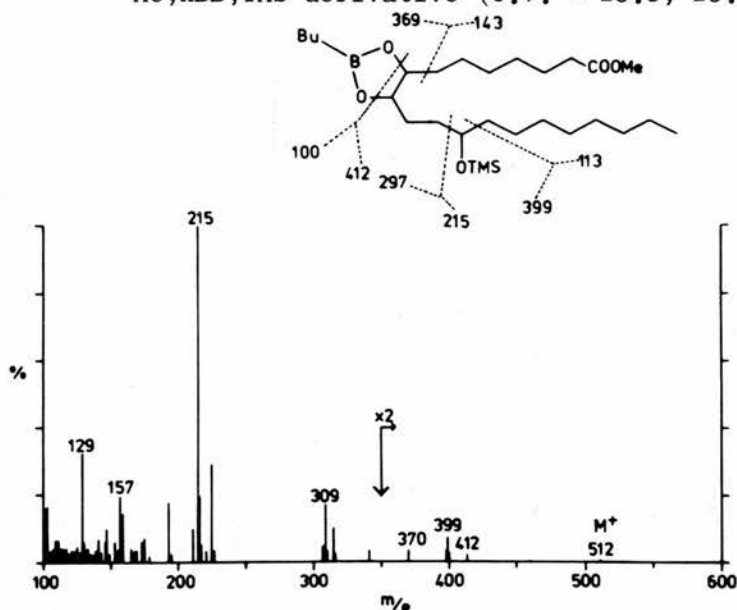


Fig. 8. M.S. of hydrogenated 8,9,12-THETA as Me,nBB,TMS derivative (C.V. = 26.0, 26.2)

Assignment of peaks was assisted by the boron isotope pattern (^{10}B 20%, ^{11}B 80%) e.g. the large peak at m/e 279 (Fig. 7) occurs by loss of 90, (TMSOH), from the 369 peak. Both these peaks contain a boron atom. The corresponding peaks in Fig. 8 are at m/e 309 and 399.

So far no biological role has been found for THETA other than the possible removal of arachidonate as a more polar metabolite. The importance of such metabolic pathways is therefore not appreciated at present, but may be considerable especially when PG biosynthesis is inhibited by drugs (indomethacin, aspirin). The quantity of THETA compared to TxB_2 formed, varies considerably even in the same subject. Washed platelets appear to give more THETA than the platelet-rich plasma incubations (Fig. 9). The best source of THETA is washed horse platelets. It is also formed by sheep and guinea-pig uterus, sheep and dog spleen. Its formation is not inhibited by aspirin-like drugs. THETA has very weak (spasmogenic) action on smooth muscle preparations, and no detectable action on human platelets themselves.

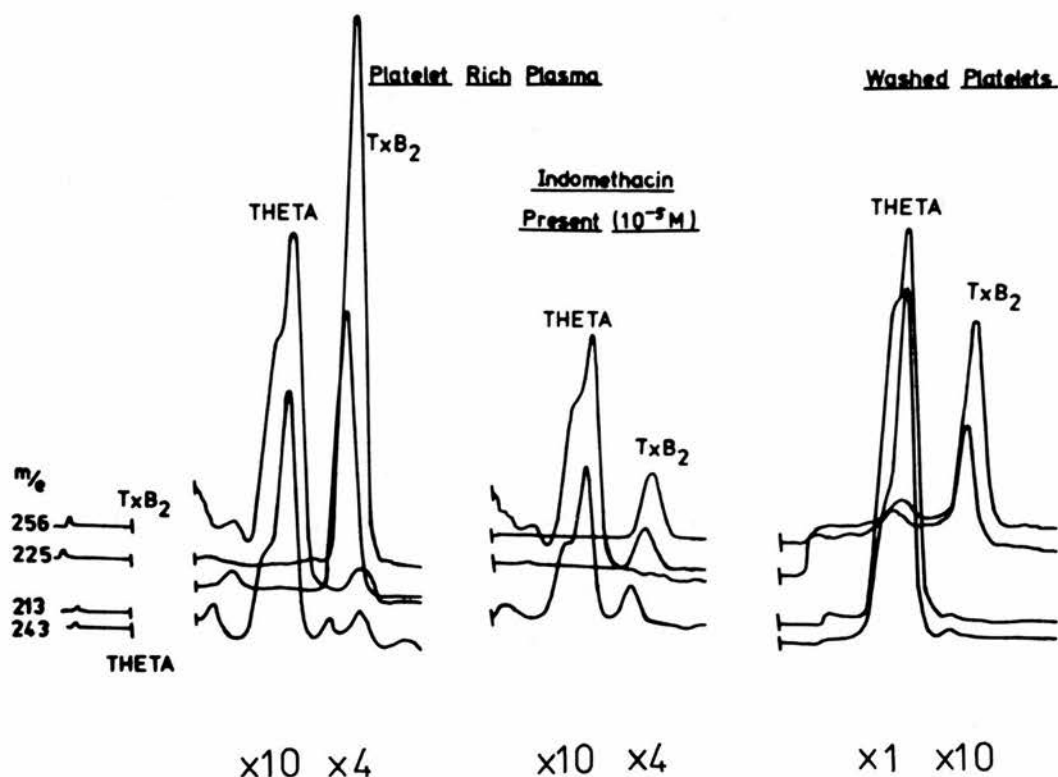


Fig. 9. Incubation of arachidonic acid with platelets under different conditions. Analysis by MID of characteristic ions.

It is of interest to note that Samuelsson and his co-workers isolated analogous trihydroxy acids from dihomogamma-linolenic acid incubations with human platelets, but were unable to establish a similar conversion for arachidonic acid (5). A mechanism suggested by the Swedish workers for the formation of such trihydroxy systems is the hydration of an epoxy-hydroxy system, derived by further oxidation of the 12-hydroperoxyeicosa-8,10,14-trienoic acid. These epoxy systems have some precedent in fatty acid oxidations (6).

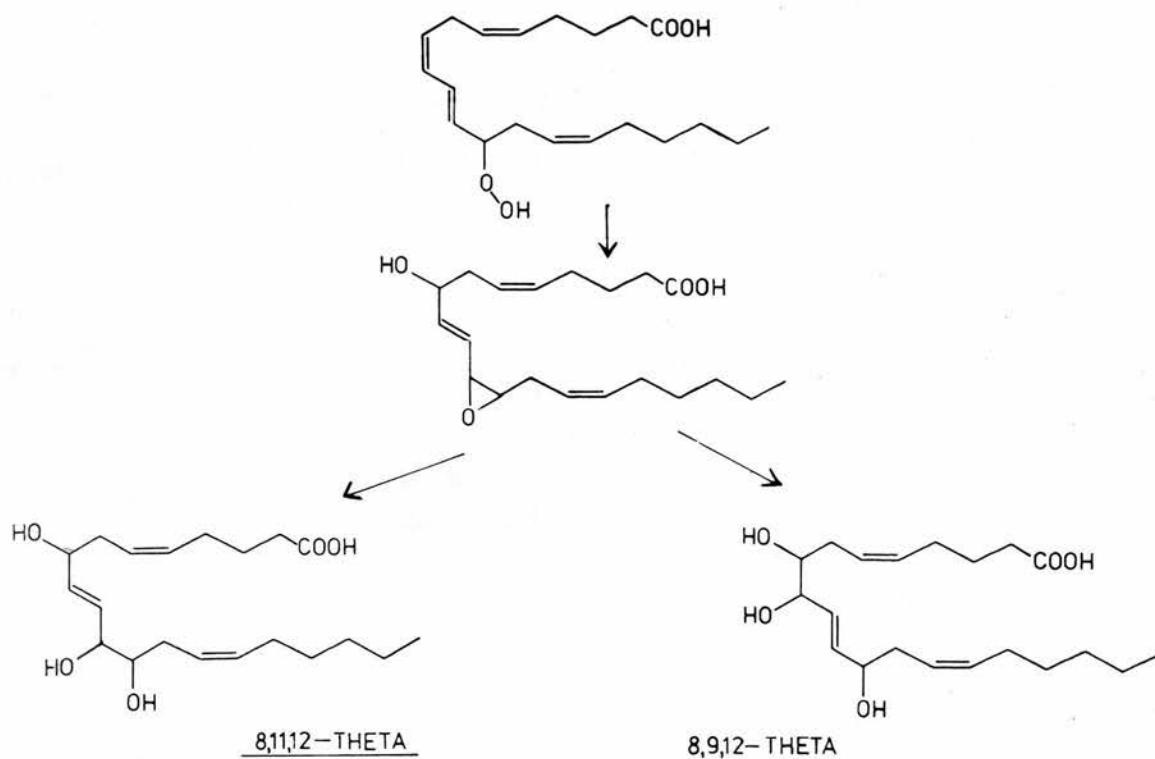


Fig. 10. Proposed mechanism of THETA formation (5)

This is not, however, the only mechanism but seems a likely possibility. Accordingly, we have looked for the intermediate epoxy compound in our analysis and we have found a possible candidate. The slightly more polar shoulder on the HETE peak in Fig. 1. appeared to be an epoxy derivative of arachidonic acid, as suggested by GC-MS data (Fig. 11). The position of the hydroxyl group has not yet been determined finally, although it is likely to be in the 8-position. The peak at m_e 269 has the constitution shown (Fig. 11) by exact mass measurement. This compound may well be the intermediate precursor to THETA. Its final chemical structure and biological actions obviously require investigation.

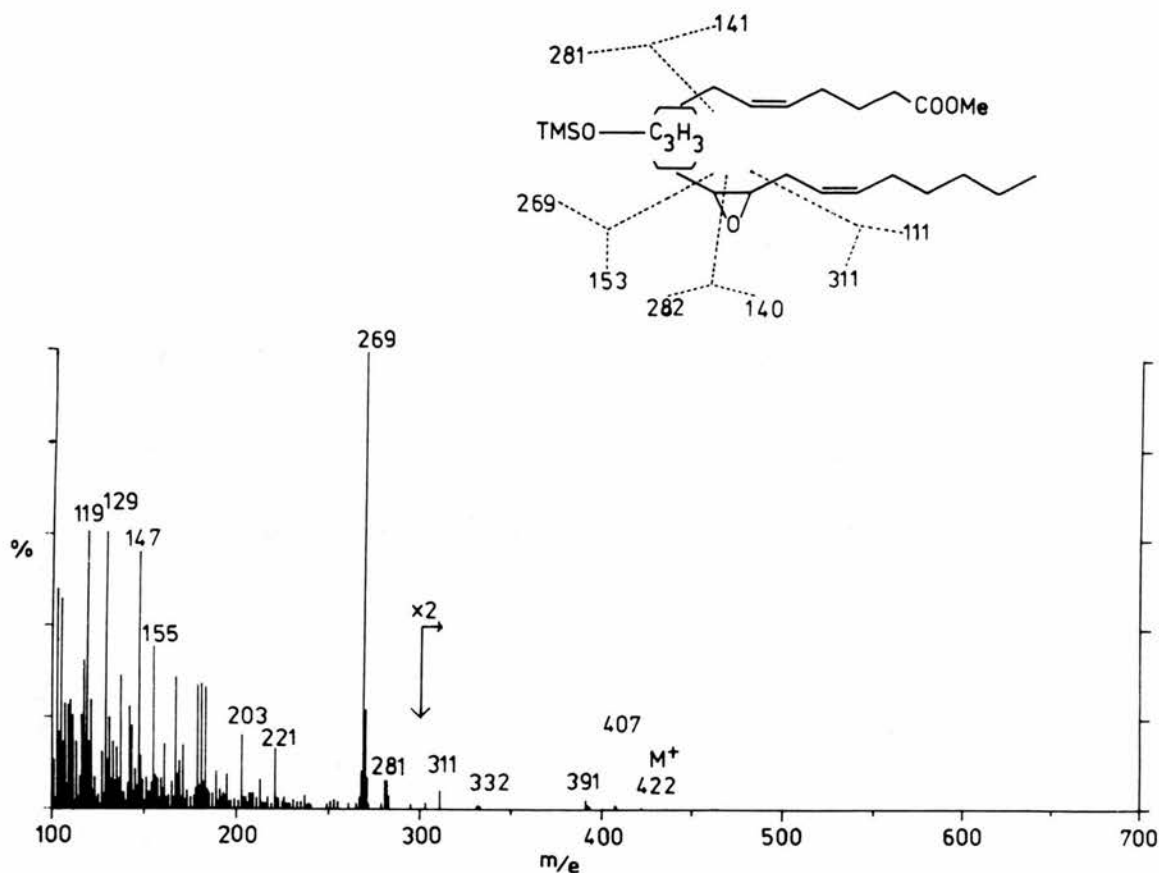


Fig. 11. Possible structure for substance in HETE fraction (Fig. 1).
Me,TMS derivative (C.V. = 22.0)

Finally we should like to speculate that these molecules, in particular the THETA precursor/s and 8,11,12-THETA could be related to the structures described by Pace Asciak some years ago (7) (Fig. 12). Detection of the first species (A) has eluded this laboratory although we have found the second substance (B) in very small quantities in the dog spleen, as indicated by its mass spectrum (Fig. 13). This substance does not form an oxime derivative, though oximation of the material extracted was required so as to shift the G.C. retention times of interfering prostanoids, for example, 6-oxo-PGF_{1α} and thromboxane B₂.

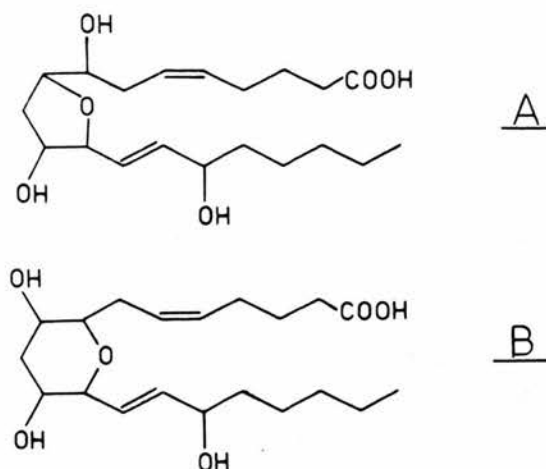


Fig. 12. Substances possibly related to THETA

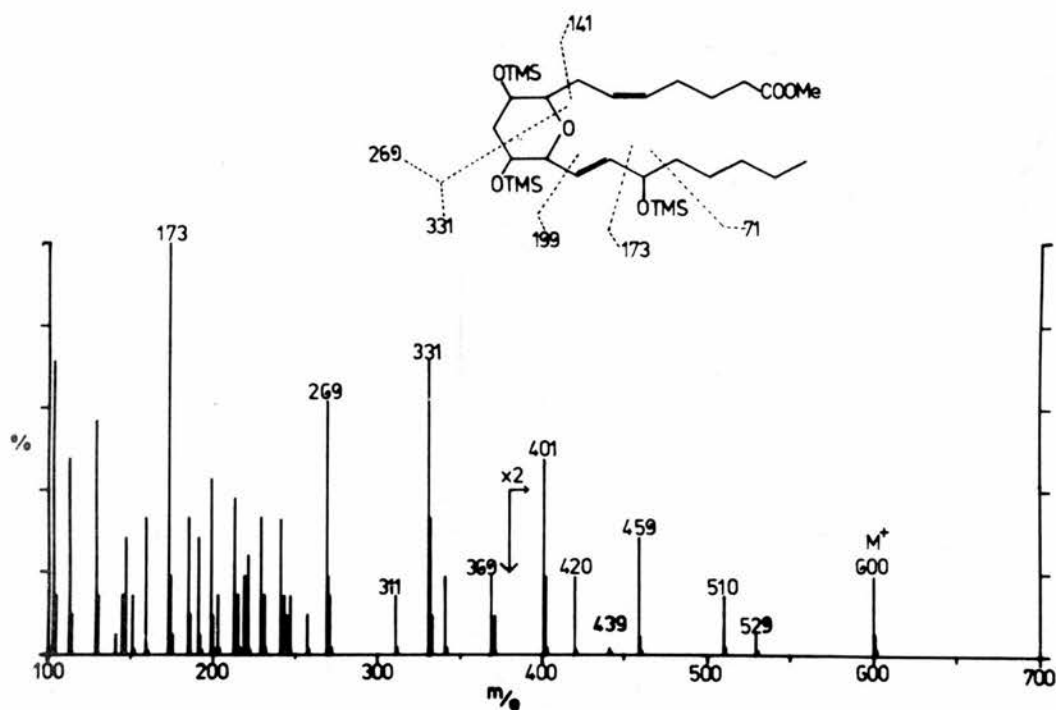


Fig. 13. M.S. of prostanoid isolated from dog spleen, 8,12-oxy-9,11,15-trihydroxy-eicosa-5,13-dienoic acid. Me,TMS derivative (C.V. = 24.2)

These structures (Fig.12) have oxygen functions in the same positions as in THETA though a further hydroxyl requires to be introduced at the 15-position. This raises the intriguing possibility of an interaction between the lipoxygenase and the cyclo-oxygenase pathways.

This work was supported by an M.R.C. grant to Professor E. W. Horton.

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THE IDENTIFICATION OF AN EPOXY-HYDROXY ACID AS A
PRODUCT FROM THE INCUBATION OF ARACHIDONIC ACID
WITH WASHED BLOOD PLATELETS

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An epoxy-hydroxy compound, 10-hydroxy-11,12-epoxy-eicosa-5,8,14-trienoic acid, has been identified as a product on incubation of arachidonic acid with washed blood platelets from human, horse, cat, dog and rabbit. Gas chromatographic - mass spectrometric (GC-MS) evidence of structure is discussed.

Introduction

Rapid metabolism of arachidonic acid occurs in human platelets by two main pathways. Fatty acid cyclooxygenase generates PGG_2 and PGH_2 which are then enzymatically isomerised to thromboxane A_2 , a very potent stimulant of platelet aggregation (1). Lipoygenase action also produces 12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (HPETE) which is subsequently transformed to 12-hydroxyeicosa-5,8,10,14-tetraenoic acid (HETE) (2). Our recent studies with washed platelets have resulted in the identification of two trihydroxy acids - 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid and 8,9,12-trihydroxyeicosa-5,10,14 trienoic acid (THETA) (3). A possible mechanism suggested for the formation of these trihydroxy acids was the conversion of HPETE to 8-hydroxy-11,12-epoxyeicosa-5,9,14-trienoic acid and subsequent hydration of this epoxide. During attempts to detect this hydroxy-epoxy intermediate an analogous compound was detected (4). Further structural identification work on this compound by GC-MS of various derivatives indicates the structure to be 10-hydroxy-11,12-epoxy-eicosa-5,8,14-trienoic acid (EPHETA).

Experimental Procedure and Results

Platelet rich plasma and washed platelets from citrated human, horse, cat, dog and rabbit blood were prepared according to Hamberg *et al* (5). Washed

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platelets (at a concentration of $5 \times 10^8 - 5 \times 10^9 \text{ ml}^{-1}$) were incubated with arachidonic acid ($1.65 \times 10^{-4} \text{ M}$) in a Krebs-Henseleit medium (containing neither calcium ions nor glucose) at 37°C for 30 min. In some experiments $1\text{-}^{14}\text{C}$ -arachidonic acid (final sp. activity $120 \text{ } \mu\text{Ci mmole}^{-1}$) was added as tracer to incubates. After acidification, the products were extracted with diethyl ether and the organic extract purified by liquid-gel partition chromatography (4). By scintillation counting of the column eluate, a more polar shoulder on the HETE peak was detected. This material was subjected to GC-MS analysis.

Gas-chromatography of the methyl ester-trimethyl silyl ether (Me/TMS) derivative showed a single peak (carbon value 22.05 on a 3% OVI column). The mass spectrum indicated a compound with molecular weight of 422 as ions were observed at m^+/e 422, 407 ($-\text{CH}_3$) and 391 ($-\text{OCH}_3$). This is consistent with a C_{20} fatty acid methyl ester having three double bonds, one trimethylsilyloxy group and one keto or epoxy oxygen. Additional significant ions were observed at m^+/e 332 ($-\text{TMSOH}$), 311 ($-\text{111}$), 282/281 ($\text{M}-140/141$), 269 (base peak). (Fig. 1).

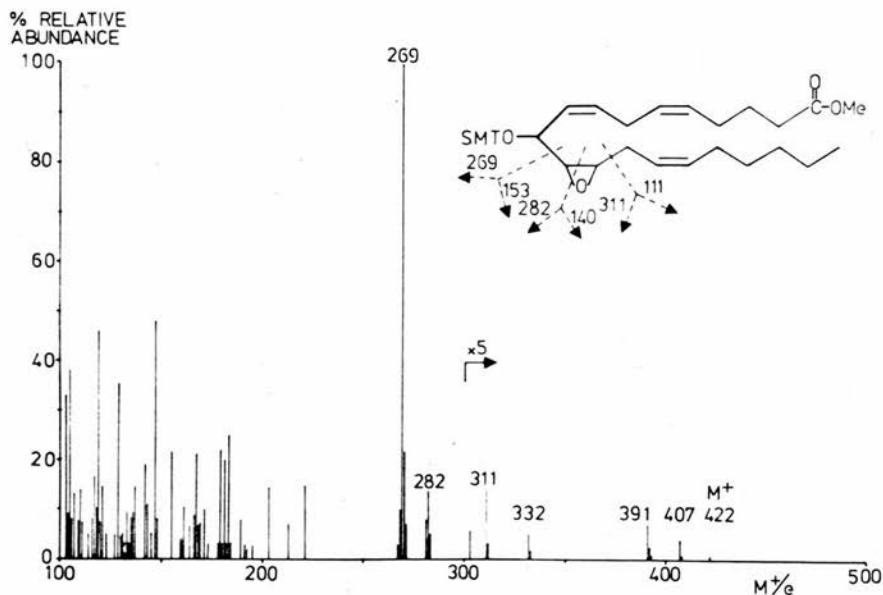


Figure 1. Mass spectrum of EPHETA (Me/TMS).

The material did not form an oxime indicating the absence of a carbonyl group. In the ethyl ester-trimethylsilyl ether derivative (carbon value 22.6) the base peak was found at m^+/e 283 suggesting that this ion and that at m^+/e 269 in the methyl ester spectrum contain the ester group. Similarly, significant ions were observed at m^+/e 296/295 suggesting that these ions and those at 282/281 in the methyl ester spectrum contain the ester group - these ions are interpreted as resulting from the transannular cleavage of the epoxide, which occurs with and without proton transfer (6). This evidence is consistent with the proposed structure of EPHETA.

Following hydrogenation (platinum oxide/methanol) and Me/TMS derivitisation the material showed two marginally separated GC peaks (C values 22.80, 22.85). The mass spectra taken at these C values were very similar and indicated the presence of three double bonds in the original material. As oleic acid epoxide was unchanged by the hydrogenation procedure it may be assumed that an epoxide group would be unaffected. Significant ions were observed at m^+/e 413 ($-\text{CH}_3$), 397 ($-\text{OCH}_3$), 315 ($-\text{H}_2$), 273 and 257.

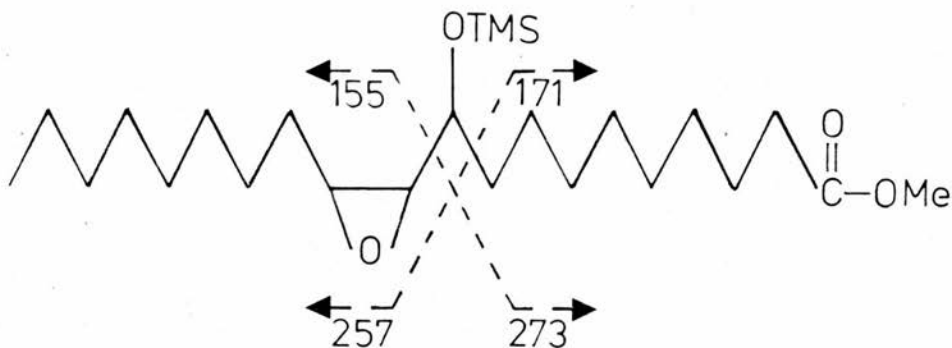


Figure 2. Fragmentation of EPHETA (Me/hydrogenated/TMS).

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The ion at m^+/e 273 confirmed the presence of two double bonds in the fragment m^+/e 269 in the Me/TMS spectrum of the original material, and the ion at m^+/e 257 was indicative of a hydroxyl group at the 10 position (Fig.2). It is possible that either hydroxyl stereoisomers or epoxy isomers of this compound are present since multiple ion detection of the major ions in the spectra also indicated two very slightly resolved peaks in the respective single ion chromatograms.

It is well known that lithium aluminium hydride (LiAlH_4) reduces an epoxide grouping to a mono-alcohol (two positional isomers are usually formed). During this reaction an ester group is also reduced to a primary alcohol. The isolated material (250 μg as the methyl ester) was therefore heated with LiAlH_4 in dry tetrahydrofuran at 60°C for 72 h. in an ampoule sealed under nitrogen. Gas chromatography of the TMS derivative of the product showed a single peak (carbon value 23.0), whose mass spectrum had significant ions at m^+/e 540 (molecular ion), 525 ($-\text{CH}_3$), 450 ($-\text{TMSOH}$), 313, 227 and 213 and was consistent with the reaction scheme shown in Fig. 3.

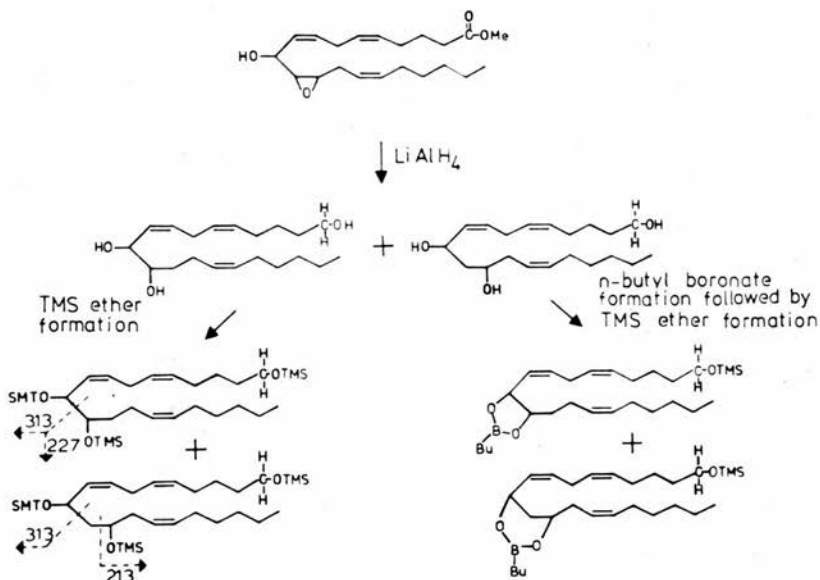


Figure 3. Reaction scheme for LiAlH_4 reduction.

The relative abundance of the ions at m^+/e 313, 227 and 213 altered in spectra taken at different positions on the GC peak, suggesting the presence of two slightly resolved products.

Gas chromatography of the *n*-butyl boronate/trimethylsilyl ether of the product of the LiAlH_4 reduction indicated that none of the TMS product remained and showed two partially resolved peaks (*C* values 23.8 and 24.2) with very similar mass spectra. Both these spectra contained the expected molecular ion, m^+/e 462, and were consistent with the proposed products (Fig. 3). The mass spectra were dominated by cleavages involving the boronate and trimethylsilyloxy groups and therefore it is not possible to tell whether the two GC peaks correspond to the two different products although this seems the most likely explanation. Since 1,2- and 1,3- diols readily form boronates, the formation of these derivatives indicates the hydroxyl group was adjacent to the epoxide in the original molecule.

All the evidence indicates the structure of this molecule as 10-hydroxy-11,12-epoxyeicosa-5,8,14-trienoic acid and not the proposed THETA intermediate 8-hydroxy-11,12-epoxyeicosa-5,9,14-trienoic acid. Further work is required to establish the configurations of the hydroxyl and epoxy groups (it is probable that the 5,6; 8,9 and 14,15 double bonds have remained *cis*). In incubations with washed platelets this molecule has been detected in smaller quantities than THETA (approximately 1/4 the amount) and on occasions in comparable amounts to thromboxane B_2 . The formation of EPHTA was not blocked by indomethacin (10^{-5}M).

A possible mechanism suggested for the formation of EPHTA is the transformation of the HPETE (at present there is no evidence that this conversion is enzymatic); analogous conversions of C_{18} fatty acid hydroperoxides have been reported (7).

Non-allylic epoxy-hydroxy acids are considerably more stable towards nucleophiles than allylic epoxy-hydroxy acids (8). It would therefore seem likely that both 8-hydroxy-11,12-epoxyeicosa-5,9,14-trienoic acid (an allylic epoxy-hydroxy acid) and 10-hydroxy-11,12-epoxyeicosa-5,8,14-trienoic acid (a non-allylic epoxy-hydroxy acid) form from HPETE. Only the more stable 10-hydroxy compound (EPHTA) accumulates whereas the less stable 8-hydroxy compound is converted to the two trihydroxy acids (THETA) by the addition of water.

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The biological properties of EPHETA are under investigation.

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Acknowledgement

These studies were supported from an M.R.C. programme grant to Professor E.W. Horton.